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INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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☐ *Additional inventors are being named on the ____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

POLYCYSTIC OVARY SYNDROME AND RELATED DIAGNOSTIC METHODS

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METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

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Respectfully submitted,

SIGNATURE Rodney D. DeKruif Date 4/22/02

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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I hereby certify that, on the date shown below, this U.S. Provisional Patent Application for "POLYCYSTIC OVARY SYNDROME AND RELATED DIAGNOSTIC METHODS" is being deposited with the United States Postal Service in an envelope addressed to the Commissioner for Patents, Box Provisional Application, Washington, DC 20231 via Express Mail No. EL803231124US on April 22, 2002.

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PROVISIONAL PATENT APPLICATION

Of

ANDREA DUNAIF

For

UNITED STATES LETTERS PATENT

On

POLYCYSTIC OVARY SYNDROME AND RELATED DIAGNOSTIC METHODS

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Evidence for Association of Polycystic Ovary Syndrome in Caucasian Women with a Marker at the Insulin Receptor Gene Locus

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ABSTRACT

The polycystic ovary syndrome (PCOS) is one of the commonest female endocrinopathies affecting 5-10% of women of reproductive age. The disorder, characterized by chronic anovulation and signs of hyperandrogenism, results from a complex interaction between genetic predisposing factors and environmental triggers. We have studied 85 Caucasian PCOS patients and 87 age-matched Caucasian control women for associations with four candidate genes: follistatin, CYP19 (aromatase), CYP17a, and the insulin receptor (INSR). These genes were analyzed using microsatellite markers located near or inside the genes. We found that only the insulin receptor gene marker D19S884 was significantly associated with PCOS (p=0.006 and even after a conservative correction p=0.042). The INSR gene region was then fine mapped with an additional panel of 9 markers but only marker D19S884, located 1 cM telomeric to the INSR gene, was again associated with PCOS. In conclusion, our results suggested that a susceptibility gene for PCOS was located on chromosome 19p13.3 in the insulin receptor gene region. It remains to be determined if this susceptibility gene is the insulin receptor gene itself or a closely located gene. Since insulin stimulates androgen secretion by the ovarian stroma it is likely that INSR function in the ovary is involved in the genetic susceptibility to PCOS.

The polycystic ovary syndrome (PCOS) is one of the commonest human endocrinopathies and affects 5-10% of women (1). The disorder is characterized by chronic anovulation, and signs of hyperandrogenism including hirsutism, alopecia and acne (2,3). Many patients also develop obesity and insulin resistance, and the syndrome is associated with greatly increased risk of type 2 diabetes mellitus (4). The etiology of PCOS is not known but epidemiologic data shows that PCOS is a familial disorder with a sibling risk ratio of 50% to 80% (5,6). Segregation analyses in families with PCOS suggested a Mendelian dominant pattern of inheritance when assuming premature baldness as the male phenotype of the disorder (7,8). However, the susceptibility genes for PCOS are unknown. Various candidate genes have been examined as possible susceptibility genes for PCOS (e.g. CYP 19, CYP17, leptin) and have given negative results (9). Recently attention has focused on the insulin gene VNTR and the follistatin gene since both were reported to be linked with PCOS (9).

In view of the strong association of PCOS with insulin resistance, the insulin receptor gene has long been thought to be a candidate gene for PCOS. Several studies have looked for mutations in the INSR gene in patients with PCOS but failed to find major mutations (10,11).

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However, genes can also be involved in the susceptibility to complex diseases with minor polymorphic changes. We have, therefore, re-examined the insulin receptor gene as well as the follistatin, CYP19 and CYP17a genes by case control association studies using microsatellite markers. Our results demonstrated a significant association of the INSR gene region with PCOS.

Subjects and Methods

Patients

We studied a total of 85 Caucasian PCOS patients. The age of the women ranged from 14 to 60 years (mean 31yrs). All the patients fulfilled the NIH consensus criteria for PCOS including: (1) a history of oligomenorrhea, (2) elevated levels of serum testosterone, and (3) exclusion of other causes of oligomenorrhea and hyperandrogenism. Forty six (54%) of the women were obese (BMI>30). The control group included 87 age-matched Caucasian women. All the control subjects had normal menstrual cycles and no clinical evidence of hyperandrogenism. For all subjects, phenotype was determined with the clinician blinded to the individual's genotype. Each participant was interviewed and examined, and signed a written informed consent before participating. All the pertinent clinical and laboratory data were recorded and stored in our data base. The project was approved by the Mount Sinai Institutional Review Board.

PCR amplification of microsatellite markers

Table 1 shows the four candidate genes tested and the markers used to test them. DNA was extracted from whole blood using the Puregene kit (Gentra Systems, Minneapolis, MN). Oligonucleotides for amplification of microsatellites were designed according to published sequences in the Genome Database (http://gdbwww.gdb.org/). Microsatellite markers were amplified as previously described (12), and the fluorescent labeled PCR products were separated using an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA), and allele calling was performed using the Genotyper™ 2.0 software. The marker data were then automatically exported to our database (Ingres™ database) where they were integrated with the already existing phenotype information and prepared for association analyses.

Statistical analysis

Association analyses were performed by the χ^2 test. P values were corrected for multiple testing by the method of Bonferoni, and a p value <0.05 after correction was considered significant. The relative risk was calculated by the method of Woolf (14).

Results

Analysis of candidate genes

Our analysis showed no association of the aromatase, follistatin, or CYP17 genes with PCOS (Table 1). In contrast, the insulin receptor gene marker D19S884 was associated with PCOS (p=0.006 and after correction for multiple testing p=0.042, Table 1).

TABLE 1. Association analysis of 4 candidate genes in PCOS patients (n=85) and controls (n=87).

Gene	Location	Markers	P value
Follistatin	5p14	D5S474	0.8871
		D5S623	0.2437
		D5S822	0.1215
Aromatase	15q21	CYP19	0.785
CYP17a	10q24.3	D10S192	0.7663
Insulin	19p13.3	D19S884	0.006*
Receptor		D19S922	0.2848

* p=0.042 after correction for multiple testing

Allele #2 was more prevalent in the controls than in the patients (9% versus 1.5%, respectively) and allele #10 was more prevalent in the patients than in the controls (33% and 22%, respectively) giving a relative risk of 1.5 to people with allele #10.

Fine mapping the insulin gene region on chromosome 19p13.3

In order to fine map the insulin receptor gene region, we analyzed an additional panel of 9 markers spanning a 7 centimorgan (cM) region flanking the insulin receptor gene. Only marker D19S884 was associated with PCOS in our cohort. None of the other markers in the region were associated with PCOS although some have been reported to be closer to the INSR than D19S884 (Table 2).

TABLE 2. Association analysis of 10 markers in the insulin receptor gene region in PCOS patients (n=85) and controls (n=87).

Marker	Location (cM)	P value
D19S216	20.0	0.891
D19S869	23.0	0.092
INSR	25.2	0.150
D19S406	25.2	0.116
D19S567	25.2	0.785
D19S873	25.2	0.475
D19S905	25.2	0.750
D19S884	26.4	0.006
D19S912	27.1	0.561
D19S922	27.2	0.178

Note: the map order and distances between the markers are all approximate and subject to change.

Discussion

Our results showed an association between PCOS and marker D19S884 in the insulin receptor gene region in a cohort of 85 Caucasian PCOS patients. This suggested that a gene affecting the phenotypic expression of PCOS was mapped to marker D19S884 located 1 cM telomeric to the insulin receptor (INSR) gene. This marker has now been found to be associated with PCOS in a family-based association study using the transmission disequilibrium test

(TDT) (13). The finding of an association of PCOS with the same INSR gene marker in two independent data sets suggests that a major gene for PCOS is located in the INSR gene region.

We found no association of PCOS with the INSR microsatellite which is located inside the INSR gene (Table 2). However, the INSR gene itself was not excluded based on this lack of association since under certain conditions, markers which are more distant from a susceptibility gene may show greater linkage disequilibrium with the disease than markers which are closer to the gene (15). This could happen if, for example, the closer marker had a higher mutation rate than the distant marker. Alternatively, the susceptibility gene for PCOS on 19p13 may be a regulatory element for the INSR gene located close to D19S884. Furthermore, the genetic maps of chromosome 19p13 are not yet exact and the location of the markers and their distances from the INSR gene are only an approximation.

Hyperinsulinism is known to play a central role in PCOS, and, therefore, the insulin receptor or a regulatory element controlling its expression are likely candidate genes for PCOS. Insulin receptors are widely distributed throughout all ovarian compartments, and insulin exerts several important effects on the ovaries including: (1) stimulation of ovarian production and secretion of androgens, (2) potentiation of the ovarian steroidogenic response to LH and FSH, and (3) suppression of apoptosis in ovarian follicles which could reduce the rates of their atresia and lead to cyst formation [for a review see (16)]. Thus, insulin actions on the ovaries, which are mediated through ovarian INSRs, may explain the characteristic findings in PCOS including hyperandrogenism, anovulation, and ovarian cyst formation. Moreover, treatment of PCOS patients with insulin sensitizers (e.g. metformin), which reduce serum insulin levels, often induce ovulation (16,17).

Previous studies have shown either normal or decreased INSR expression in the ovaries in PCOS patients when compared with controls (16). In addition, several workers have analyzed the INSR gene for mutations in PCOS patients. Early reports demonstrated mutations in the tyrosine kinase domain of the insulin receptor gene in a few PCOS patients with severe insulin resistance (18-20). In contrast, others were not able to find missense or nonsense mutations in the INSR gene in insulin resistant PCOS patients (10,11,21). It is unlikely that the common variety of PCOS is caused by major mutations in the INSR, such as found in the severe insulin resistance of the type A syndrome (22), and more likely that single nucleotide polymorphisms (SNP's) in the INSR, or in regulatory elements controlling its expression, are involved in the genetic susceptibility to PCOS. SNP's would induce a less profound change in INSR function and/or tissue expression.

In summary, we found evidence that a susceptibility gene for PCOS was located in the insulin receptor gene region. It was possible that this gene mediated its effects by influencing INSR gene expression at the ovary. Since insulin stimulates androgen secretion by the ovarian stroma it is likely that changes in the INSR or its signaling pathway in the ovary could explain some of the genetic susceptibility to PCOS. Identification, of the PCOS susceptibility gene in the INSR gene region may shed further light on the participation of insulin and its receptor in the pathogenesis of PCOS.

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1 A. SPECIFIC AIMS

A1. How this Project Contributes to the Theme of the SCOR. Polycystic ovary syndrome (PCOS) is among the most common endocrine disorders in premenopausal women, affecting 7-10% of this population. It is characterized by hyperandrogenism, chronic anovulation and, frequently, obesity. We have shown that PCOS confers a substantially increased risk for impaired glucose tolerance (IGT) and type 2 diabetes mellitus (DM2), with prevalence rates of glucose intolerance approaching ~40%. Women with PCOS have profound insulin resistance as well as pancreatic β-cell dysfunction, independent of obesity and glucose intolerance. However, skeletal muscle insulin resistance reverses in cultured myotubes suggesting that insulin resistance in this tissue is induced by factors in the *in vivo* environment. We have recently shown that hyperandrogenemia is the reproductive phenotype in males as well as female relatives of PCOS women. Moreover, Urbanek and colleagues have shown (Project 2) that this phenotype appears to have a genetic basis in PCOS families and shows significant linkage and association with a marker locus on chromosome 19p in the region of the insulin receptor (allele 8 of D19S884). These findings have been replicated in a second sample of families and in an independent case-control study. We now have extremely exciting evidence that this allele is also associated with a metabolic phenotype in PCOS probands and their brothers: increased post-challenge glucose levels, apparent defects in insulin secretion, especially in response to sulfonylurea, and accelerated weight gain with age. Abbott (Project 3) has shown that *in utero* testosterone excess can reproduce many features of the PCOS reproductive and metabolic phenotype in female rhesus monkeys, including decreased insulin secretion and increased LH levels. Levine (Project 4) has shown that one mechanism for these changes is androgen-mediated suppression of ATP-sensitive potassium (K^+_{ATP}) channels. These channels also mediate glucose- and sulfonylurea-stimulated insulin secretion by the pancreatic β-cells. Taken together, these observations have led to a new hypothesis for the etiology of PCOS: genetic variation resulting in hyperandrogenemia results in many of the reproductive and metabolic features of PCOS by fetal androgen programming. In this Project, we will test two components of the hypothesis. First, is the metabolic phenotype that is associated with the marker locus decreased insulin secretion, consistent with androgen-mediated suppression of K^+_{ATP} channel activity? Second, is there *in utero* androgen excess, decreased fetal insulin secretion and/or intrauterine growth retardation (IUGR) in the female offspring of PCOS women, and does the marker allele identify a subpopulation of offspring with these findings? This clinical research Project will complement the basic studies by providing evidence in PCOS women to support the overall hypothesis of this SCOR.

A2. Aim 1. To test the hypothesis that homozygous or heterozygous PCOS carriers of the marker allele, allele 8 [A8(+)] of D19S884, are more insulin resistant than women without this allele [A8(-)].

We will determine whether A8(+) PCOS women have defects in insulin action compared to A8(-) PCOS women. This aim will be accomplished by examining the sensitivity and responsiveness to insulin of glucose disposal, free fatty acids (FFA) flux and endogenous glucose production (EGP) during sequential multiple insulin dose euglycemic clamp studies.

A3. Aim 2. To test the hypothesis that A8(+) PCOS women have more profound defects in insulin secretion compared to A8(-) PCOS women.

We will directly measure insulin secretion in response to various secretagogues to determine whether it is decreased in A8(+) PCOS women compared to A8(-) PCOS women. This aim will be accomplished by examining pancreatic islet function with an oral glucose tolerance test (OGTT), meal tolerance test, graded glucose infusion, glucose-dependent arginine stimulation test and tolbutamide-modified frequently sampled intravenous glucose tolerance test (FSIGT).

A4. Aim 3. To test the hypothesis that there is *in utero* testosterone excess, altered insulin secretion and/or intrauterine growth retardation in the female offspring of PCOS women. To determine whether A8(+) female offspring have more profound changes in these parameters compared to A8(-) female offspring.

We will measure androgen and insulin levels in amniotic fluid from pregnant PCOS compared to pregnant control women. We will also measure androgen and insulin levels in cord blood. Further, we will assess gestational age in

PCOS offspring to that in offspring of matched control women. We will determine whether any of these parameters differ in A8(+) compared to A8(-) [redacted] offspring.

B. Background

B1. Etiology of PCOS: Evidence for a Genetic Susceptibility. It is possible to show experimentally that androgen excess or increased GnRH release can reproduce the PCOS reproductive phenotype. It is also clear that extreme insulin resistance secondary to mutations in the insulin receptor gene can cause the PCOS reproductive phenotype (1). Accordingly, intense debate is ongoing regarding the primary defect that initiates the syndrome. Nevertheless, there is well-documented familial clustering of PCOS that provides evidence for a genetic susceptibility to the disorder (1)(2). Although some studies have suggested that there is an autosomal dominant mode of inheritance, these studies have been limited by a lack of prospective design, a failure to examine many first-degree relatives, and an unknown phenotype, except in reproductive age women (2-4). PCOS is more likely a complex genetic disease with at least several major susceptibility genes (5). We have shown that the intermediate reproductive phenotype of hyperandrogenemia aggregates in PCOS families (6). Moreover, PCOS first-degree relatives with this reproductive phenotype also exhibit evidence of insulin resistance (Preliminary Studies). Thus, identifying genes associated with the reproductive abnormalities may also identify genes contributing to insulin resistance. In support of this hypothesis, we have identified an allele of a marker in the region of the insulin receptor, A8 of D19S884, that is both linked and associated with the reproductive phenotype (Preliminary Studies and Project 2) (7;8). We used a family based association test for the association analysis, the transmission disequilibrium test (TDT), which tests for association in the presence of linkage and controls for population stratification (9-11). Further, we have replicated the association in a second sample of families. The association of this marker with PCOS has been confirmed in an independent case-control study, and the marker allele is associated with intermediate metabolic phenotypes (Preliminary Studies)(8;10;12).

B2. Rationale for Association Studies with Marker Locus. Since association is dependent on the presence of linkage disequilibrium, and linkage disequilibrium is maintained over relatively short genetic distances, the evidence for association in the TDT analysis suggests that D19S884 is close to the PCOS susceptibility gene (9). Nevertheless, a considerable amount of time may be necessary to identify the susceptibility gene that is in linkage disequilibrium with D19S884 (Project 2)(13;14). Accordingly, we plan to investigate the association of metabolic phenotypes with A8. Associations of a phenotype with a marker locus rather than a gene have been demonstrated in studies of maturity onset diabetes of the young (MODY). Such studies defined these MODY metabolic phenotypes well before the gene linked to the marker locus was positionally cloned (15-17). The association between quantitative metabolic phenotypes and anonymous chromosomal markers has also been investigated in diabetes genome scans (18;19). Moreover, the presence of an association between the marker locus and a metabolic phenotype provides additional evidence for a susceptibility gene near the marker locus (18;19).

Only three candidate genes for PCOS have been identified in linkage studies: CYP11a (cholesterol side-chain cleavage enzyme), the insulin gene variable number of tandem repeats (VNTR) and follistatin. There has been linkage and association using family-based analyses with an allele of the insulin gene VNTR locus and insulin levels in PCOS (20-22). Our further studies of follistatin and CYP11a have not supported a major role for variation in either of these genes in susceptibility to PCOS (7)(23). We failed to find evidence for an association between the insulin VNTR and PCOS in our family studies (6). Other putative candidate genes for PCOS have been identified in case-control studies (1;2). Polymorphisms in insulin receptor substrate (IRS)-1, IRS-2, PPAR-γ pro12ala allele have been associated with metabolic phenotypes in a recent case-control studies (6;24;25). However, case-control studies must be interpreted with caution since they are particularly susceptible to false positive results due to population stratification (5;11;26-28). Therefore, we are going to focus our efforts on the D19S884 region since A8 is both linked and associated with reproductive and metabolic phenotypes in PCOS. As additional genes or marker loci that meet these stringent criteria are discovered, we will also investigate their association with the phenotypic features of PCOS.

B3. PCOS is a Unique Subphenotype of Insulin Resistance. There is profound peripheral insulin resistance in PCOS similar in magnitude to that seen in DM2 (29). However, the mechanism of insulin resistance in PCOS

differs from that seen in DM2 or obesity (30;31). We have shown that serine-phosphorylation of the insulin receptor (IR) is caused by an extrinsic serine kinase and results in decreased IR signaling in cultured PCOS fibroblasts (32). The presence of a serine kinase inhibiting IR phosphorylation in PCOS fibroblasts has been confirmed recently in an independent laboratory (33). Post-IR signaling defects are selective, affecting metabolic but not mitogenic pathways in PCOS fibroblasts (34). There are post-IR signaling defects in PCOS skeletal muscle, and preliminary studies suggest that these also impair metabolic but not mitogenic pathways (Preliminary Studies)(31). Further, the pattern of changes in signaling proteins in skeletal muscle differs from that in other insulin resistant conditions such as DM2, obesity and gestational diabetes (31). PCOS skeletal muscle does not exhibit significant differences in the abundance of the IR, IRS-1, or the p85 regulatory subunit of phosphatidylinositol-3 (PI3)-kinase. The abundance of IRS-2 is increased suggesting that this change is compensatory for decreased IRS-1 mediated signaling.

B4. Skeletal Muscle Insulin Resistance is an Acquired Defect in PCOS. Skeletal muscle is the major target tissue on a quantitative basis for insulin-mediated glucose disposal (IMGD) *in vivo*, accounting for 85% of glucose utilization in the fed state (35). Based on our studies in PCOS fibroblasts, we hypothesized that insulin resistance was a genetic defect and that skeletal muscle would have persistent defects in insulin action as a stable phenotype in culture, similar to findings in DM2 (36). To test this hypothesis, we examined insulin action in cultured myotubes from PCOS and control women (Preliminary Studies). In contrast to cultured skeletal muscle from DM2, we found no evidence for intrinsic decreases in insulin sensitivity in PCOS cultured skeletal muscle (37). However, PCOS cultured skeletal muscle was not entirely similar to control because there were significant increases in basal, non-insulin-mediated glucose uptake and constitutive activation of mitogen-activated protein kinase (MAPK) pathways. Activation of MAPK was also present in PCOS muscle biopsies indicating this finding was not an artifact of tissue culture conditions. These increases in MAPK activity are another unique feature of the PCOS insulin resistance phenotype and are not seen in DM2 (38). **These findings suggest that the primary defect in PCOS is <u>not</u> skeletal muscle insulin resistance.**

B5. Tissue Differences in Insulin Action in PCOS. The studies in fibroblasts and skeletal muscle indicate that there are tissue differences in insulin action; similar findings have been reported in mice with disruption of insulin signaling pathways (39). In contrast to our findings in skeletal muscle, McAllister has reported constitutive increases in p38 stress-activated MAPK and decreased p44MAPK in passaged PCOS theca cells (40). Ciaraldi and colleagues first proposed that such tissue differences in insulin action may account for continued insulin actions on the ovary in the face of resistance to insulin's metabolic actions (41). The selective nature of insulin resistance in PCOS with preservation and even enhancement of growth-related MAPK pathways may also contribute to reproductive actions of insulin in the face of resistance to its metabolic actions.

B6. Mechanisms for Acquired Defects in Insulin Action in PCOS. This observation of reversible skeletal muscle defects in insulin action has led to a re-formulation of our hypothesis for the pathogenesis of insulin resistance in PCOS. We now propose that a circulating factor causes insulin resistance *in vivo* in PCOS. Candidate factors include FFA, cytokines such as tumor necrosis factor (TNF)-α or resistin, and androgens (42-44). There is an increasing body of evidence from human and animal studies to support the hypothesis that skeletal muscle insulin resistance in various settings is an acquired defect (42;44;45). Further, we propose that intrinsic alterations in skeletal muscle (e.g. activation of MAPK) increase susceptibility to the insulin-resistance inducing effects of the circulating factors, and we have shown that cultured PCOS skeletal muscle has increased susceptibility to FFA-mediated insulin resistance (Preliminary Studies) (46).

B7. Androgens and Insulin Resistance in PCOS. Androgens represent an obvious circulating factor that could produce acquired defects in insulin action in PCOS. Women with upper-body obesity share many features of PCOS, such as insulin resistance, increased subcutaneous abdominal adipocyte size and abnormalities in the regulation of lipolysis (47). Since women with upper-body obesity often have increased androgen production, it is possible that androgens are a common final path for these metabolic defects in PCOS and upper-body obesity (48;49). However, the hypothesis that androgens play a major role in the pathogenesis of insulin resistance in PCOS has been largely discounted because suppressing androgens does not normalize insulin action in PCOS (50;51). Further, suppressing androgens does not alter resistance to ß-adrenergic receptor agonists in isolated adipocytes in PCOS (52;53).

B8. Adiposity-Related Insulin Resistance in PCOS. It has been suggested that adiposity accounts for insulin resistance in PCOS (54). In Scandinavian PCOS women, insulin sensitivity could be completely normalized by weight reduction (55). However, abnormalities in insulin secretion persisted (55). In obese PCOS women matched to control women for visceral fat, no significant differences in insulin sensitivity or EGP existed (56). In contrast, we have found that lean PCOS women matched to control women for total fat mass and waist:hip ratios (WHR) had significantly decreased IMGD (57;58). However, increases in visceral adipose tissue (VAT) can escape detection with anthropometric measurements, so it remains possible that these lean PCOS women had increased VAT (59). Few studies have quantitated VAT in PCOS, and there are conflicting reports as to whether it is increased compared to control women matched for total fat mass (52;56;60;61). Lean PCOS women have increased abdominal fat cell size, a correlate of increased visceral adipose mass (47;57). We also found a synergistic negative effect of adiposity and PCOS on EGP, suggesting that adiposity had a greater impact in PCOS than in reproductively-normal women (57;58). Holte and colleagues reported similar findings for insulin sensitivity (54). Increased VAT could, in turn, be a consequence of androgen programming (47;62).

B9. FFA in PCOS. The classic candidate mediators of adiposity-related insulin resistance are FFA (63;64). However, the importance of FFA in the pathogenesis of insulin resistance in women has been challenged recently (65). A study has demonstrated gender differences in susceptibility to FFA-mediated peripheral insulin resistance: men were susceptible to this FFA action whereas women were not(65). We have exciting evidence from cultured skeletal muscle that supports the presence of such differences in susceptibility to FFA-mediated insulin resistance (Preliminary Studies). PCOS skeletal muscle is more susceptible to this FFA action than cultured skeletal muscle from control women (Preliminary Studies). This mechanism could account for the greater deleterious effect of adiposity on insulin action that has been observed in PCOS (54;57). Circulating FFA levels have not been well-studied in PCOS, nor are we aware of studies of FFA flux in the disorder (66). A recent study found that lipolysis was increased in PCOS visceral fat (67). This difference could lead to increased portal FFA levels, which in turn could induce hepatic and peripheral insulin resistance (63). FFA levels could also be increased in PCOS because of decreased suppression of lipolysis due to the relative decreased insulin secretion that is also found in the syndrome (64). It remains possible that other fat-cell derived factors, adipokines, such as TNF-α, contribute to adiposity-related insulin resistance in PCOS.

B10. Possible Fetal Origins of PCOS. The fetal origins or Barker hypothesis proposes that IUGR as evidenced by low birth weight causes insulin resistance, cardiovascular disease and other features of the insulin resistance syndrome (68;69). Decreased fetal nutrition is proposed to result in decreased fetal insulin secretion and growth. Insulin resistance is a compensatory mechanism that further decreases fetal nutrient use: the "thrifty" phenotype. Extensive animal studies support the long-term impact of the fetal environment on the adult animal, known as fetal programming (69). The molecular mechanisms for these phenomena remain largely unknown, but permanent alterations in gene expression produced by changes in gene methylation may play a role (70). Many epidemiologic studies in humans support the association between low birth weight and metabolic diseases (68;69;71;72). To our knowledge, the Barker hypothesis has not been tested prospectively in humans. However, the long-term consequences, such as obesity and glucose intolerance, of fetal hyperinsulinemia, which results in high birth weight in the offspring of diabetic mothers, have been documented by our Co-Investigator, Boyd Metzger, and his colleagues(73). Thus, it is clear in humans that there are permanent physiologic alterations related to the intrauterine environment.

Sex steroids are well known to produce sex-specific differentiation in a number of fetal tissues, such as the urogenital tract and the brain (64). Less appreciated are the programming actions of androgens that alter metabolism. Transient exposure to androgens in several animal models can permanently decrease insulin sensitivity and secretion, as well as hepatic clearance of insulin (64). Androgens can also alter body fat distribution and lipolysis (64). Thus, androgen programming can recreate many feature of the PCOS metabolic phenotype including insulin resistance, β-cell dysfunction, catecholamine resistance in subcutaneous abdominal adipocytes, but increased visceral adiposity and sensitivity to catecholamine-mediated lipolysis in this fat depot (62;74;75). Prenatally androgenized female rhesus monkeys are also smaller for gestational age (Project 3). Indeed, prenatally androgenized monkeys have many of the reproductive features of PCOS: increased LH levels, irregular ovulation,

polycystic ovaries and functional ov[illegible]n hyperandrogenism (62;76;77). Some of [illegible]ndrogen programming effects depend on the sex of the animal (78[illegible]

There is evidence for fetal origins of some features of PCOS in human studies. Ibanez and colleagues have reported that both girls with elevated adrenal androgen levels or with PCOS were significantly smaller for gestational age than reproductively normal control girls(79;80). It is intriguing to speculate that androgen programming could account for the recently reported sex differences in susceptibility to FFA-mediated insulin resistance and explain why women with PCOS appear to have the male phenotype for this effect (65). Drs. Abbott and Levine will directly investigate the programming effects of androgens in their Projects (3 and 4). We will investigate whether there is any evidence of *in utero* androgen excess in PCOS as part of this Project.

B11. Significance. This Project will determine the specific mechanism of the metabolic phenotype associated with the A8 marker locus. Such information will be critical for understanding the function of the putative gene in this region once it is positionally cloned (Project 2). Even before the gene is cloned, it will be possible to use the marker locus to identify women at risk for metabolic consequences of PCOS. This Project also has important implications for understanding the genetic basis of DM2 since PCOS is a leading cause of DM2 in women and male relatives also appear to be affected. Finally, this Project will provide evidence to support (or refute) a novel new hypothesis for the pathogenesis of PCOS. Understanding the genetic basis and pathophysiology of PCOS will lead to new therapies and, perhaps, to cure of this common and overarching women's health.

C. PRELIMINARY STUDIES

All subjects fulfilled the criteria outlined in Section D4 of this proposal and studies were performed in age-, weight- and ethnicity-comparable cases and controls. No subject had DM2, and control subjects had normal glucose tolerance. Approximately 30% of PCOS women had IGT, but none had impaired fasting glucose.

C1. Familial Aggregation of Hyperandrogenemia and Insulin Resistance in PCOS Families. The evidence to support the genetic analysis of a complex trait is familial aggregation. This finding was present for hyperandrogenemia in PCOS kindreds with 46% of sisters thus affected. Only one-half of these sisters fulfilled diagnostic criteria for PCOS -with chronic anovulation (≤ 6 menses/year) and hyperandrogenemia. The remaining affected sisters had a novel phenotype: hyperandrogenemia (HA) with regular menses. There was a significant bimodal distribution of testosterone (T) levels in the sisters whereas the distribution was unimodal in the control women (Figure 1). The bimodal distribution was consistent with a monogenic trait controlled by two alleles of an autosomal gene (6). This study strongly suggested that hyperandrogenemia in PCOS had a genetic basis and that a possible candidate gene would be one involved in the regulation of both ovarian and adrenal steroidogenesis since levels of the adrenal androgen dehydroepiandrosterone sulfate (DHEAS) were also increased.We determined whether familial aggregation of metabolic defects was present in first-degree relatives. To control for the confounding effects of ethnicity on insulin sensitivity, we limited the population to Non-Hispanic White women (81). Two hundred seventeen sisters of 165 PCOS probands and 47 ethnically-comparable, reproductively-normal control women were studied. Phenotypes were defined as PCOS: ≤ 6 menses/yr and an elevated total or biologically available (u) (T) level; HA: menses every 27-35 d and an elevated T or uT level; Unaffected (UA): menses every 27-35 d and normal T, uT, and DHEAS levels (Table 1). We concluded that there was familial aggregation of insulin resistance in PCOS consistent with a genetic trait (82). Hyperandrogenemia and insulin resistance track together suggesting that they may reflect variation in the same gene or in closely linked genes (see below).

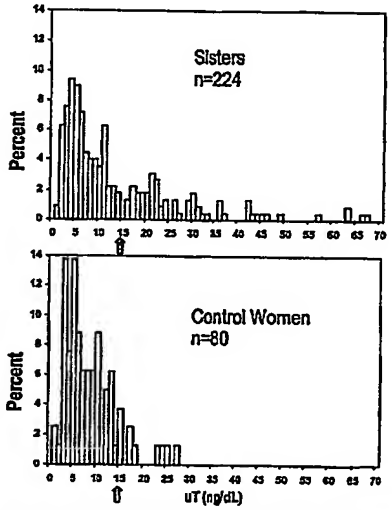


Figure 1. Distribution of uT levels. A uT level of 15ng/dL is 2 SD above the control mean and a value>15ng/dL was used to diagnose hyperandrogenemia. The distribution of uT levels is significantly bimodal (P<0.001) in the sisters, whereas it is not in the controls.

We determined whether a male [redacted]notype was pres[redacted] in the brothers of PCOS women (83). One hun[redacted]d nineteen brothers of 87 unrelated women with PCOS and 68 weight- and ethnicity-comparable unrelated control men were studied. We did not find premature male balding in the brothers (a suggested male phenotype in previous studies). Brothers of women with PCOS had significantly elevated DHEAS (3035 ± 1132 brothers vs 2492 ± 1172 ng/mL control men, P<0.05). There was a significant positive linear relationship between DHEAS levels in PCOS probands and their brothers (r=0.35, P=0.001). We concluded that the PCOS brothers appeared to have a reproductive phenotype with elevated DHEAS levels. The elevated DHEAS levels might reflect the same underlying abnormality in steroidogenesis for which we have found evidence in ~50% of the premenopausal sisters of PCOS women.

Table 1. Metabolic Parameters in [redacted] of PCOS Probands (mean ± SD)

	PCOS	HA	UA	Control	P
Glucose mg/dL	89 ± 9[c]	83 ± 8	87 ± 8[c]	84 ± 8	<0.001
Insulin µU/mL	24 ± 11[a]	19 ± 12[a]	14 ± 7	14 ± 8	<0.001
Glucose:Insulin Ratio	4.6 ± 2.3[a]	5.6 ± 2.6[a]	7.3 ± 2.6[b]	7.5 ±3.5	<0.001

[a] significant vs UA and control; [b] significant vs control; [c] significant vs HA and control; [d] significant vs HA and UA; [e] significant vs HA, UA, and control

Fasting glucose levels did not differ in brothers compared to controls. Both fasting insulin (16 ± 9 brothers vs 14 ± 8 µU/mL controls, P=0.07) and proinsulin (15 ± 12 vs 11 ± 6 pmol/L, P=0.08) levels tended to be higher in the PCOS brothers, but this difference did not achieve statistical significance in this sample. The proinsulin:insulin molar ratio, a marker of β-cell function, was not increased in the brothers. There were significant positive correlations between both insulin levels (r= 0.27, P<0.05) and proinsulin levels (r=0.54, P<0.001) in brothers and their proband sisters with PCOS, suggesting that these were also heritable traits in PCOS families. Total TTG levels were significantly increased in PCOS brothers (191 ± 153 brothers vs 144 ± 95 controls mg/dL, P< 0.05). There were no significant differences in cholesterol, HDL or LDL levels. These findings also suggested that the brothers of PCOS women had insulin resistance and lipid abnormalities associated with the insulin resistance syndrome.

2. Genetic Analyses. We determined whether there was linkage between polymorphic markers at candidate genes and the combined phenotype of PCOS or hyperandrogenemia (PCOS/HA) with an affected sib pair (ASP) analysis (7). We tested for association in the presence of linkage with the TDT analysis in PCOS proband-parent trios. We screened 37 candidate genes (33 chromosomal locations) involved in steroidogenesis, gonadotropin secretion, insulin action, or energy metabolism in 168 families and 39 affected sib pairs (ASP). The ASP and TDT analyses were performed by Dr. Margrit Urbanek, Principal Investigator on Project 2. We found significant evidence for linkage with follistatin (identity by descent [IBD]=72%, χ^2=12.97, nominal P=3.2 X 10^{-4}, P<0.01 corrected for multiple tests) and for association with markers in the region of the IR by the TDT. However, the association findings in the region of the IR were not significant after a very stringent correction for multiple testing at the time of that publication (see below). We concluded that follistatin (FS) and the IR were high priority candidate genes for PCOS.

We investigated the possibility of genetic variation in FS (23). Such variation might lead to overexpression or increased binding activity of FS that could contribute to the pathogenesis of PCOS by resulting in arrested folliculogenesis, increased thecal androgen secretion, decreased FSH release, and decreased insulin secretion. We found no evidence for sequence variants that play a major role in PCOS. We found a nominally significant association of a single nucleotide polymorphism in exon 6 by the TDT analysis that did not remain significant after correction for multiple tests. There were no differences in FS expression in PCOS fibroblasts. We concluded that variation in FS did not play a major role in PCOS.

In the original series the marker D19S884 in the region of the IR showed the strongest evidence for association in 168 trios in the TDT analysis, χ^2=8.53, although this result was not significant after using a Bonferroni correction for testing ~350 alleles (7). However, in our second data set of 190 trios, D19S884 still has the strongest evidence for association, χ^2=8.84, as well as in the combined data set of 358 trios, χ^2=12.95, P=3.21x10^{-4} (8). There is now also evidence for linkage in the IR region with IBD= 63%, χ^2=8.784, P=3.04 x 10^{-3} in 98 ASP (8). In addition, a case-control study also found that an allele of D19S884 was significantly associated with PCOS, providing support of our findings in an independent sample (12). We are performing simulation analyses to determine empirically the significance of our association findings (84).

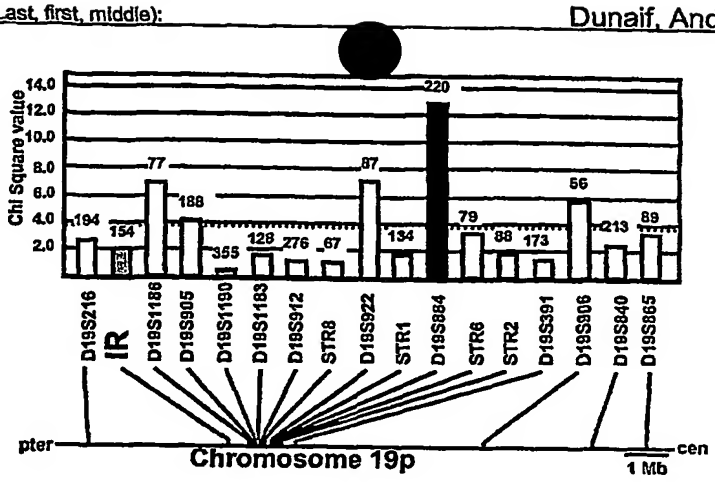


Figure 2. TDT analysis of chromosome 19p, D19S884 χ^2=12.95, P=3.21x10^{-4} with 220 transmissions. Solid bar DS19S884, gray bar IR, dotted line χ^2=4, nominal P=0.05.

However, this association finding has been replicated in our second sample of families as well as in an independent case-control study, and there is also evidence for linkage using the ASP analysis. The evidence for association in the TDT analysis suggests that D19S884 is in linkage disequilibrium with the PCOS susceptibility gene. Taken together, these findings provide strong evidence to implicate a gene close to D19S884 in susceptibility to PCOS (11). Moreover, since the required sample to detect "signal" is inversely related to the increase in disease susceptibility conferred by a gene, these significant findings in a fairly small sample size suggest that this locus contains a major susceptibility gene for PCOS (84).

The marker D19S884 maps to 1 Mb centromeric to the IR, and linkage disequilibrium is usually maintained only over much smaller regions (85). A gene other than the IR most likely accounts for the association of D19S884 (figure 2). It remains possible that this gene may still regulate IR function or expression or that it may be another gene involved in insulin action. Alternatively, this gene may be related to other biologic pathways in PCOS. There are several intriguing known genes in this region: 1) SCYA25, a thymus-expressed cytokine (86), 2) MAP2K7, a mitogen activated serine/threonine kinase that activates c-Jun N-terminal kinase (JNK) in response to activation by growth factors, cytokines and stress (87) and 3) resistin, a recently identified cytokine, which is expressed in adipocytes, down-regulated by thiazolidinediones, and that induces insulin resistance in rodents (88).

Our studies in PCOS cultured skeletal muscle (see below) suggest that an extrinsic factor produces insulin resistance in this tissue *in vivo*, and cytokines, such as resistin or SCYA25, are excellent candidates. MAP2K7 is a candidate serine kinase for the increased serine phosphorylation we have identified in PCOS fibroblast IRs (32). These potential mechanisms are examples of how our cellular and molecular studies can suggest which candidate genes should be given priority for further study. We have investigated whether resistin is associated with PCOS. We typed a SNP in the 5' untranslated region of the resistin gene and used the TDT test for association in 258 families. We did not find any evidence for association with PCOS in general, in PCOS women who were obese (BMI>27 kg/m^2), or in PCOS women who were insulin resistant (glucose:insulin ratio <4.5, see above) (manuscript in preparation). We are intensely searching for the PCOS susceptibility gene near D19S884, but identifying it may take a considerable amount of time (Project 2), as has been the case for certain of the maturity onset diabetes of the young (MODY) genes and NIDDM1 (i.e. calpain 10) (14;89;90). Nevertheless, we can investigate the metabolic phenotype associated with allelic variation at this marker even before the susceptibility gene is identified (see below) (15;16;17).

C3. Genotype - Phenotype Analy[illegible] (Table 2, Figures 2-4). We have investigated whether th[illegible]COS/HA allele, A8 of D19[illegible]84, was associated with any metabolic phenotypic feat[illegible]s in PCOS women. To control for the confounding effect of obesity and ethnicity on insulin action, we limited the population to obese Non-Hispanic White individuals (81). We combined homozygous and heterozygous carriers of D19S884 A8 in the A8(+) group. Approximately 30% of PCOS women are A8(+) by this definition. The frequency of allele 8 Centre d'Etude du Polymorphisme Humain (CEPH) families was 20.6%. Control women were age-, ethnicity-, and BMI-comparable, reproductively-normal women (n=64). We had data on 75 g OGTT 0 h and 2 h glucose and insulin responses in 32 A8(+) PCOS and 81 A8(-) PCOS (Table 2). We performed FSIGT studies in 6 A8(+) PCOS and 16 A8(-) PCOS. In the OGTT group, A8(+) PCOS women were significantly younger than the A8(-) group so all analyses were adjusted for age by analysis of covariance (ANCOVA). There was a significant age*BMI interaction ($P<0.05$), and BMI increased with age in A8(+) and remained stable in A8(-). A similar trend was found for blood pressure but did not achieve statistical significance ($P=0.06$). There were no significant differences in fasting glucose or insulin levels or in glucose:insulin ratios. These parameters were significantly increased in both A8(+) and A8(-) PCOS compared to age-, weight-, and ethnicity-comparable, reproductively-normal control women. This result is consistent with the presence of similar degrees of insulin resistance in A8(+) and A8(-) PCOS women. However, 2 h post-challenge glucose levels were significantly increased in A8(+) PCOS compared to A8(-) PCOS and to control women ($P<0.05$, ANCOVA) (Table 2, Figure 3C). Post-challenge insulin levels did not differ in the A8(+) and A8(-) PCOS groups and were higher than in the control women; this result is consistent with the presence of insulin resistance in both A8(+) and A8(-) groups (Table 2, Figure 3D). The adrenal androgen DHEAS tended to be higher in the A8(+) women, but this difference did not achieve statistical significance (data not shown). There were no other significant differences in hormonal parameters in the A8(+) and A8(-) PCOS groups. The A8(+) and A8(-) PCOS women who had FSIGTs were well matched for age and BMI (Table 2). They had virtually identical insulin sensitivity (SI) values indicating that they had similar degree of insulin resistance. However, glucose levels were slightly higher and insulin levels substantially lower in the A8(+) PCOS (Figure 3A and 3B). The striking differences in insulin responses during the FSIGT are depicted by the shaded area in Figure 3B. The observation that insulin responses to tolbutamide were much lower in A8(+) PCOS women suggests that there may be a defect in the sulfonylurea receptor (91). Dr. Levine has evidence to indicate that this defect may be an androgen-medicated action on K^+_{ATP} channels in the ß-cell (Project 4). When expressed as area-under-the-curve (AUC) insulin:glucose, this difference approached statistical significance ($P=0.09$) in this very small sample of A8(+) and A8(-) PCOS women (Table 2).

Table 2. Non-Hispan[illegible]te Obese PCOS (mean ± SEM)

	A8(+) (n)	A8(-) (n)	P
AGE yr	27 ± 1 (82)	30 ± 1 (160)	0.008
BMI kg/m²	37.4 ± 0.8 (82)	38.4 ± 0.6 (160)	0.3 [a,b]
Systolic mm/Hg	126 ± 2 (60)	122 ± 1 (119)	0.06 [a,b]
Diastolic mm/Hg	76 ± 1 (60)	75 ± 1 (118)	0.2 [a]
0 h Glucose mg/dL	93 ± 2 (81)	91 ± 1 (160)	0.2 [a]
2 h Glucose mg/dL	153 ± 8 (32)	137 ± 4 (82)	0.03 [a]
0 h Insulin µU/mL	29 ± 2 (81)	29 ± 1 (158)	1.0 [a]
2 h Insulin µU/mL	168 ± 16 (31)	167 ± 14 (80)	0.5 [a]
Proinsulin pmol/L	25 ± 2 (77)	22 ± 1 (152)	0.3 [a]
FSIGT			
AGE yr	26 ± 3 (6)	27 ± 1 (16)	0.9
SI $x10^{-4}$/min/µU/mL	2.0 ± 0.5 (6)	2.0 ± 0.5 (16)	1.0
SG $x10^{-2}$	2.1 ± 0.2 (6)	1.8 ± 0.1 (16)	0.3
DI $x10^{-3}$/min	115 ± 23 (6)	126 ± 24 (16)	0.8
AIRg µU/mL	57 ± 14 (6)	67 ± 5 (16)	0.4
AUC Insulin $_{2\text{-}10}$ µU/mL	680 ± 105 (6)	806 ± 57 (16)	0 3
AUC Glucose $_{0\text{-}180}$ mg/dL	19729 ± 1424 (6)	18222 ± 574 (16)	0.2
AUC Insulin $_{0\text{-}180}$ µU/mL	10615 ± 2871 (6)	15254 ± 2869 (16)	0.2
AUC Insulin:Glucose $_{0\text{-}180}$	0.51 ± 0.10 (6)	0.81 ± 0.13 (16)	0.09

[a]ANCOVA Adjusted for Age; [b]Interaction

We investigated whether there [redacted] phenotypic differences in A8(+) (n[redacted]) and A8(-) (n=39) obese brothers of PCOS probands. The groups were well matched for age and BMI. Proinsulin levels, proinsulin:insulin molar ratios and TTG levels were significantly increased in A8(+) brothers compared to A8(-) brothers (Figure 4). HDL levels tended to be lower in A8(+) brothers (35±2 A8(+) vs 40±2 A8(-) mg/dL, P=0.053). Very few brothers had OGTTs so responses could not be analyzed. DHEAS levels tended to be higher in the A8(+) brothers (data not shown).

To determine whether A8 had an impact on metabolic parameters in normal individuals, we examined A8(+) (n=20) and A8(-) (n=26) obese unaffected sisters as defined above. There were no significant differences in fasting metabolic parameters, but too few sisters had OGTTs so changes similar to those in the PCOS sisters may have escaped detection. We also plan to perform these analyses in A8(+) vs A8(-) parents. We had too few individuals who were homozygous for A8 in any of the groups to study the impact of gene dosage. Most of the A8(+) individuals were heterozygous.

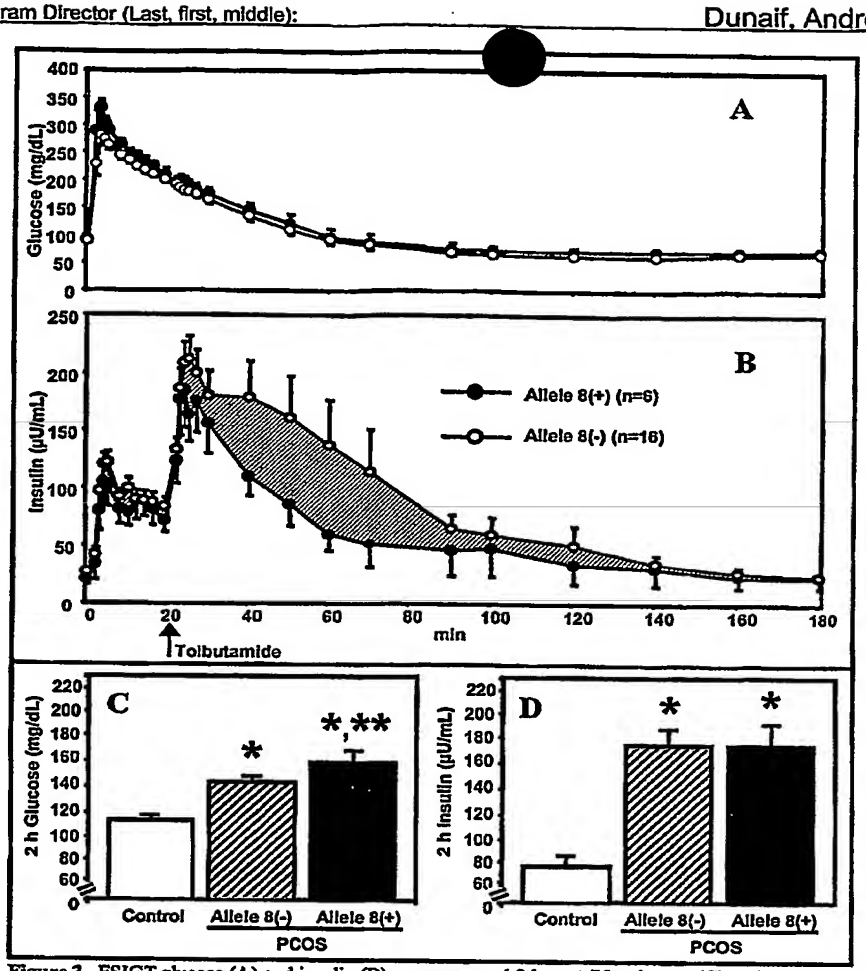


Figure 3. FSIGT glucose (A) and insulin (B) responses and 2 h post-75g glucose (C) and insulin (D) levels in obese A8(+) and A8(-) PCOS women. Tolbutamide, 500mg iv, given at 20 min of the FSIGT. The shaded area in panel B is the difference in insulin responses in A8(+) vs A8(-) PCOS. *P<0.05 vs weight matched control women, **P<0.05 vs A8(-) PCOS, by ANCOVA adjusted for age.

We have identified significant changes in BMI with age and in post-challenge glucose levels in A8(+) PCOS women. The lack of similar increases in post-challenge insulin levels suggests a failure of compensatory insulin secretion in the A8(+) PCOS. An independent test of insulin action, the FSIGT, performed in a small subset of these A8(+) PCOS also suggests decreased insulin secretion. The magnitude of insulin resistance appears to be similar in the A8(+) and A8(-) PCOS. However, OGTT parameters of insulin action are relatively insensitive, and the sample size for the FSIGT was quite small. Accordingly, both insulin secretion as well as action needs to be assessed directly to determine the metabolic impact of A8. Further, the association of increasing BMI with age was found in cross-sectional data and needs to be confirmed in prospective studies.

The A8(+) brothers also appear to have a metabolic phenotype consistent with the insulin resistance syndrome (92;93). The increase in proinsulin levels and the proinsulin:insulin molar ratio in A8(+) brothers suggests that they may also have β-cell dysfunction (94;95). These

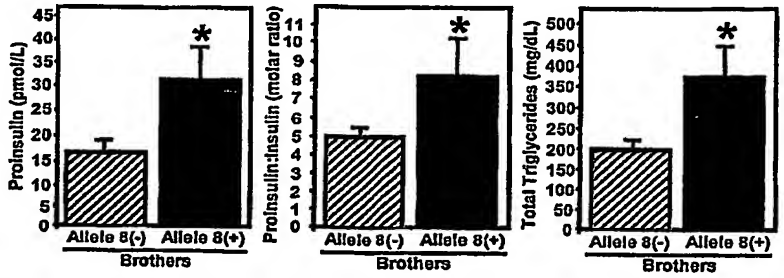


Figure 4. Fasting proinsulin, proinsulin:insulin and total triglyceride levels in obese A8(-) and A8(+) brothers of PCOS women, *P<0.05.

findings are similar to the trends th[redacted]e noted in the comparison of PCOS brothe[redacted]control men (see above) and now achieve statistical significance[redacted]n the population is stratified by A8 status. This very important result may pro[redacted] a way to identify the affected brothers of PCOS women, i.e. those who are A8(+). Proinsulin levels were high[redacted] in A8(+) brothers (Figure 4) than in A8(+) PCOS probands (Table 2) suggesting that there may be sex differences in the metabolic phenotype. We had insufficient data to determine whether A8 was associated with a metabolic phenotype in the reproductively unaffected A8(+) sisters.

We recognize that we have performed a number of statistical tests and that some of the significant differences may represent false positive results. Nevertheless, both OGTT and FSIGT responses as well as the results in different study groups have all provided similar data. Taken together, these associations between A8 and metabolic phenotypes in PCOS women and male first-degree relatives provide strong support for the hypothesis that a gene in the region of D19S884 plays an important role in insulin action and/or secretion. There appear to be additional susceptibility genes for insulin resistance in PCOS since A8(-) PCOS women also have evidence for defects in insulin action.

C4. Acquired Insulin Resistance in PCOS Skeletal Muscle (Table 3). We performed this study to determine whether the defects that we detected in acutely isolated skeletal muscle were intrinsic. Myoblasts were harvested from Bergstrom needle biopsies of the vastus lateralis and grown in primary culture using the method of Henry et al.(36;37;96;97). There were no significant differences in population doubling time or cell number in PCOS compared to control myoblasts. There were no significant differences in the fold-stimulation of glucose transport or glucose incorporation into glycogen (Table 3). There were significant increases in basal glucose transport (Table 3) in PCOS compared to control. This finding may be explained by significant increases in GLUT1 abundance in PCOS. GLUT4 abundance was similar in PCOS and control myotubes. Metabolic signaling pathways were similar in PCOS and control myotubes whereas mitogenic pathways were upregulated in PCOS. Basal mitogen-activated protein kinase kinase (MEK) phosphorylation tended to be increased, and insulin-stimulated MEK phosphorylation was significantly increased in PCOS without a change in MEK abundance. Consistent with this activation of MEK, p44/42 MAPK phosphorylation (detected by an antibody that recognizes p44 and p42 MAPK only when dually phosphorylated at thr202 and tyr204; the p44 and p42 bands were quantitated together) was significantly increased at baseline and in response to insulin in PCOS without any change in the abundance of these signaling proteins. There was a significant increase in p44/42 MAPK phosphorylation at baseline in PCOS skeletal muscle biopsies (63±9 PCOS n=8 vs 30±6 n=8 control, % internal standard, $P<0.05$) without changes in MAPK abundance.

Table 3. PCOS and Control Cultured Myotubes (mean ± SEM)

	Control (n=8)	PCOS (n=7)	P
Glucose transport			
Basal nmol/mg/min	13.4 ± 1.3	19.8 ± 2.2	0.02
100 nM	19.0 ± 2.1	26.8 ± 3.2	0.06
Fold	1.4 ± 0.4	1.35 ± 0.04	NS
Glycogen synthesis			
Basal nmol/mg/h	4.7 ± 0.5	7.0 ± 1.2	NS
100 nM	12.1 ± 1.5	19.4 ± 4.5	NS
Fold	2.6 ± 0.1	2.7 ± 0.2	NS
Protein abundance			
IRβ*	87 ± 38	93 ± 13	NS
IRS-1*	154 ± 30	253 ± 46	0.07
IRS-2*	69 ± 11	76 ± 15	NS
p85*	136 ± 52	118 ± 36	NS
GLUT 1*	106 ± 32	184 ± 48	0.02
GLUT 4*	96 ± 7	96 ± 8	NS
Tyrosine phosphorylation			
IRβ 100 nM*	34 ± 6	39 ± 4	NS
IRS-1 100 nM*	42 ± 15	32 ± 11	NS
PI3-kinase activity			
IRS-1 - associated			
Fold	17 ± 2	18 ± 6	NS
IRS-2 - associated			
Fold	8 ± 1	9 ± 1	NS
Phospho MAPK p44/42*			
Basal	28 ± 10	94 ± 9	0.003
100 nM	68 ± 23	206 ± 35	0.02
MAPK*	122 ± 22	108 ± 13	NS
Phospho MEK*			
Basal	49 ± 45	88 ± 20	NS
100 nM	118 ± 45	293 ± 47	NS
MEK*	196 ± 32	157 ± 32	0.04

*% internal standard

There were constitutive increases in glucose uptake, GLUT1 abundance and p44/42 MAPK activation in PCOS myotubes. The increase in MAPK phosphorylation in skeletal muscle biopsies indicates that the findings in cultured myotubes are not an artifact of the culture conditions. Activation of growth related MAPK pathways has not been found in other insulin resistant states and is another unique feature of the PCOS phenotype (36;37;38;96;98). The p44/42 MAPK pathway is activated by growth factors such as insulin and regulates cell proliferation, cell survival and gene expression (99;100). Enhanced signaling through these pathways, basally and in response to insulin, may contribute to some of the PCOS phenotype.

C5. Mechanisms for Acquired D[redacted]ts in Insulin Action in PCOS: Role of [redacted] and TNF-α in PCOS. We con[redacted]e that decreases in IMGD and IRS-1-associated PI3-kinase activity in PCOS resolve in cultured skeletal mus[redacted] suggesting that these defects are acquired secondary to *in vivo* environment (31). Candidate factors that could modulate insulin sensitivity include androgens, FFA, TNF-α, resistin and adiponectin (42;43). Fasting FFA levels were significantly increased in obese PCOS (n=8) compared to control (n=7) women of comparable age and weight (434± 46 control vs 607 ± 58 PCOS μmol/L, P<0.05), despite higher fasting insulin levels (13 ± 2 control vs 22 ± 5 PCOS μU/mL) in PCOS, a finding consistent with resistance of FFA suppression by insulin *in vivo*. The FFA levels were similar to those in women with upper-body obesity (101). TNF-α levels were not significantly increased in obese PCOS (n=20) compared to weight- comparable control (n=12) women (7 ± 2 control vs 6 ± 1 PCOS pg/mL) in contrast to prior reports (102;103).

It is also possible that there is increased sensitivity to FFA or TNF-α actions in PCOS. To investigate this hypothesis, we examined the impact of incubating cultured myotubes from PCOS (n=7) and control (n=7) women with the FFA palmitate (0-1 mM) for the last 48 h during the 4 d differentiation process or with TNF-α (0-25 ng/mL) for 2 h. (104-107). Palmitate caused a significantly greater decrease in both basal as well as insulin-stimulated glycogen synthesis in PCOS than in control (both P<0.05), whereas TNF-α had a similar effect to decrease glycogen synthesis in PCOS and control myotubes. These findings were presented at the 61st Scientific Sessions of the American Diabetes Association, June 2001, abstract 1107P, and the manuscript is in preparation (46).

C6. Relevant Publications

1. Legro RS, D Finegood, **A Dunaif.** A fasting glucose to insulin ratio is a useful measure of insulin sensitivity in women with polycystic ovary syndrome. J Clin Endocrinol Metab 83:2694-2698, 1998.

2. Legro RS, D Driscoll, JF Strauss, J Fox, **A Dunaif.** Evidence for a genetic basis for hyperandrogenemia in the polycystic ovary syndrome. Proc Natl Acad Sci USA 95:14956-14960, 1998.

3. Legro RS, A Kunselman, WC Dodson, **A Dunaif.** Prevalence and predictors of risk for type 2 diabetes mellitus and impaired glucose tolerance in polycystic ovary syndrome: A prospective, controlled study in 254 affected women. J Clin Endocrinol Metab 84:165-169, 1999.

4. Urbanek M, RS Legro, DA Driscoll, R Azziz, DA Ehrmann, RJ Norman, JF Strauss III, RS Spielman, **A Dunaif.** Thirty-seven candidate genes for polycystic ovary syndrome; strongest evidence for linkage is with follistatin. Proc Natl Acad Sci USA. 98:8573-8578, 1999.

5. Martens JWM, DH Geller, W Arlt, RJ Auchus, VS Ossovskaya, H Rodriguez, TD Tisty, **A Dunaif,** WL Miller. Enzymatic activities of P450c17 stably expressed in fibroblasts from patients with the polycystic ovary syndrome. J Clin Endocrinol Metab, 85:4338-4346, 2000.

6. Urbanek M, X Wu, KR Vickery, L-C Kao, LK Christneon, RS Lego, DA Driscoll, JF Strauss III, **A Dunaif,** RS Spielman. Allelic variants of the follistatin gene in polycystic ovary syndrome. J Clin Endocrinol Metab 85:4455-4461, 2000.

7. **Dunaif A,** E Diamanti-Kandarakis, A Lee, X Wu. Defects in insulin receptor signaling in vivo in the polycystic ovary syndrome (PCOS). Am J Physiol Endicronol Metab 281: E392-399, 2001.

8. Rui L, V Aguirre, JK Kim, GI Shulman, A Lee, A Corbould, **A Dunaif,** MF White. Phosphorylation of ser307 in IRS-1 mediates feedback and heterologous inhibition of insulin signaling. J Clin Invest 107:181-189, 2001.

9. Legro RS, AR Kunselman, **A Dunaif.** Prevalence and predictors of dyslipidemia in women with polycystic ovary syndrome. Am J Med, 111:607-13, 2001.

10. Palmert MR, CM Gordon, A Kartashov, RS Legro, SJ Emans, **A Dunaif.** Screening for abnormal glucose tolerance in adolescents with polycystic ovary syndrome. J Clin Endocrinol Metab, 87:1017-23, 2002.

11. Legro RS, M Urbanek, AR Kunselman, BE Leiby, **A Dunaif**. Self selected women with polycystic ovary syndrome are reproductively and metabolically abnormal. Fertil Steril, in press, 2002.

12. Legro RS, AR Kunselman, L Demers, SC Wang, R Bentley-Lewis, **A Dunaif**. Elevated dehydroepiandrosterone sulfate levels as the reproductive phenotype in the brothers of women with polycystic ovary syndrome. J Clin Endocrinol Metab, in press, 2002.

13. Legro RS, R Bentley-Lewis, D Driscoll, SC Wang, **A Dunaif**. Insulin resistance in the sisters of women with polycystic ovary syndrome: association with hyperandrogenemia rather than menstrual irregularity. J Clin Endocrinol Metab, in press, 2002.

C7. Hypothesis – Figure 5

1. It is our hypothesis that variation in a gene regulating steroidogenesis results in hyperandrogenemia. This androgen excess in turn causes metabolic abnormalities in PCOS.

Finding an A8 associated metabolic phenotype will support this hypothesis (Aims 1 and 2).

2. This steroidogenic abnormality leads to increased androgen production by the fetal ovary and adrenal. The resulting intrauterine androgen excess results in increased LH release and decreased insulin secretion (Project 3).

Finding increased fetal androgen and/or decreased fetal insulin levels in A8(+) offspring will support this hypothesis (Aim 3).

3. Androgens alter LH release and insulin secretion by changing the activity of K^+_{ATP} channels in GnRH neurons and pancreatic β-cells (Project 4).

Finding decreased sulfonylurea-stimulated insulin secretion in A8(+) PCOS (Aim 2) and decreased fetal insulin levels in A8(+) offspring will support this hypothesis (Aim 3).

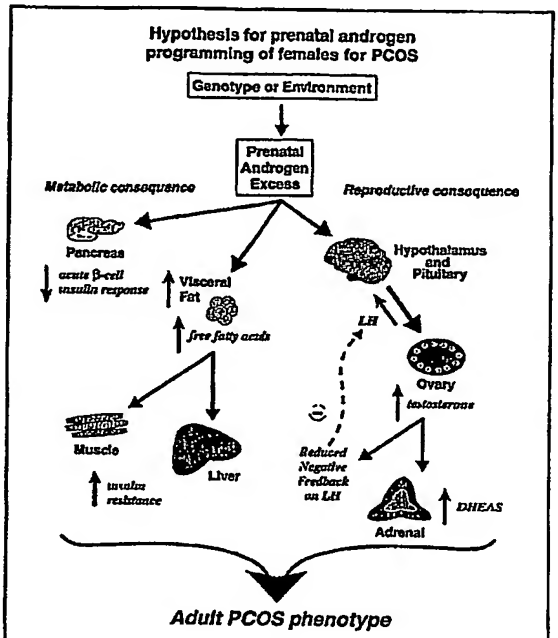


Figure 5. Hypothesis for genetic variation resulting in androgen excess, which causes metabolic and reproductive defects by prenatal programming.

4. Androgens also program adipose tissue resulting in increased visceral adiposity and increased sensitivity of these adipocytes to catecholamines-mediated lipolysis (47;52;108;109). These changes result in increased FFA delivery to the liver, which increases hepatic glucose production (63). Intrauterine androgen programming decreases hepatic clearance of insulin and alters muscle insulin action (78;110).

Finding evidence for increased visceral fat, increased FFA flux, decreased suppression of endogenous glucose production and decreased insulin clearance in A8(+) PCOS women will support this hypothesis (Aims 1 and 2).

5. A8 was identified in linkage studies with the reproductive phenotype of hyperandrogenemia. In adults, however, there are no significant differences in androgen levels in A8(+) compared to A8(-) PCOS women (Preliminary Studies). We propose that A8 results in prenatal androgen excess.

Finding increased fetal androgen levels in A8(+) offspring will support this hypothesis (Aim 3).

6. Insulin sensitivity appears to be similar in A8(+) and A8(-) adult PCOS (Preliminary Studies). We propose that the major effect of A8 is on insulin secretion and that we propose that additional genetic factors contribute to insulin resistance in PCOS since defects in insulin action are present in A8(-) PCOS (Preliminary Studies).

Finding similar defects in insulin-m[redacted]ed glucose disposal in A8(+) and A8(-) P[redacted] will support this hypothesis (Ai[redacted]).

D. RESEARCH DESIGN AND METHODS (subject selection and detailed methods follow the Study Protocols)

D1. Aim 1. To test the hypothesis that homozygous or heterozygous PCOS carriers of the marker allele, allele 8 [A8(+)] of D19S884, are more insulin resistant than women without this allele [A8(-)].

a. Rationale. We will test for metabolic phenotypic differences corresponding to allelic variation at the D19S884 marker, A8, which is both linked and associated with PCOS, in Aims 1 and 2. This approach has been employed successfully to characterize phenotypes in MODY1 and MODY3 well before the genes at the marker locus were identified (15-17;111). This approach has also been used to investigate linked intermediate metabolic phenotypes in genome scans for DM2 (18;19). The presence of an associated phenotype provides additional evidence that the marker is in linkage disequilibrium with a susceptibility gene (5).

We could also assess the impact of a variety of other genetic variants that have been associated with metabolic phenotypes in PCOS (e.g. IRS-1, IRS-2) and in the general population (e.g. β3-adrenergic receptor, PPAR-γ) (24;27). Further, we may identify new PCOS susceptibility loci in our family studies. However, each additional gene/locus that we investigate increases the probability of false positive results (5). These analyses need to be corrected for multiple testing, a step which will decrease our statistical power. Accordingly, we will investigate only variants where there is considerable evidence to support a potential role in PCOS. We may gain some insight into the overall role A8 plays since we will genotype all of our control subjects for A8 and will assess whether there are any differences associated with A8(+) status.

Our preliminary studies suggest that A8(+) PCOS women do not differ in terms of insulin sensitivity compared to A8(-) PCOS women. However, this finding needs to be verified with direct measurement of insulin action with the glucose clamp technique (112). If A8(+) PCOS women have decreased insulin secretion, they may also have increased FFA flux and EGP compared to A8(-) PCOS women(113). These parameters will also be measured directly. A sequential multiple dose euglycemic clamp will be performed so that insulin sensitivity as well as maximal responsiveness can be examined (57). Our preliminary studies also suggest that A8(+) PCOS women may gain weight more quickly than A8(-) women (Preliminary Studies). Thus, it is possible that this allele is associated with alterations in adiposity and body composition will be directly measured (114).

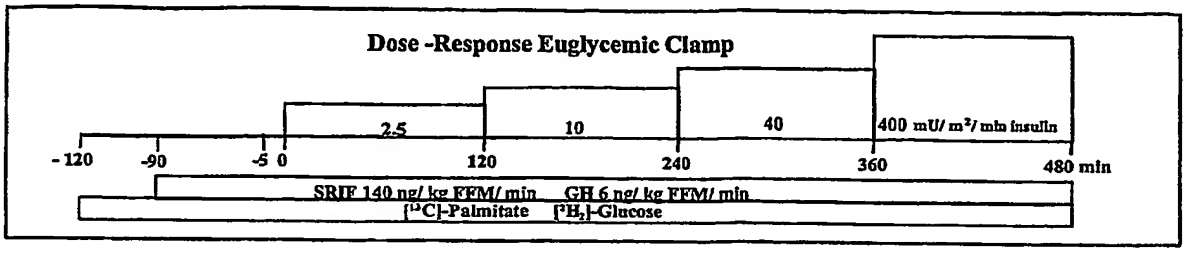
b. Experimental Approach. We will determine body composition and fat topography on all subjects so that we can match subjects on these parameters. Our previous studies may have failed to detect differences in suppression of EGP by insulin in lean PCOS compared to lean control women because we did not study sufficiently low doses of insulin (57). Accordingly, insulin doses of 2.5, 10, 40 and 400 mU/m^2/min will be administered. The 2.5 mU/m^2/min insulin dose will produce steady-state insulin levels of ~40 pM to permit the assessment of EGP sensitivity to insulin (57). The 10 mU/m2/min insulin dose will produce steady-state insulin levels of ~150 pM (57). We have previously determined that maximal rates of insulin-mediated glucose disposal are achieved with an insulin dose of 400 mU/m2/min (57). EGP will be measured using [6,6-2H]-glucose and steady-state tracer kinetics (57;115).

Somatostatin (SRIF) will be administered to suppress endogenous insulin secretion so that insulin levels can be matched across study groups (116;117). GH will be replaced because it has an important impact on lipolysis and because differences in GH levels have been reported in PCOS compared to control women (118;119). Glucagon will not be replaced to avoid hyperglycemia and because differences in glucagon levels and the suppression of glucagon by insulin have not been found in PCOS (A Dunaif, unpublished observations) (56). However, glucagon levels will be determined during the study, and glucagon will be replaced if differences in glucagon levels in PCOS compared to control women are detected. It will be necessary to allow only 2 h for the glucose tracer to achieve steady-state because all subjects will have fasting euglycemia (57).

c. Protocol Study 1

i. Subjects. Age- and weight-matched A8(+) and A8(-) PCOS and control women

ii. Visit. Sequential multiple dose euglycemic clamp



d. Endpoints. Total fat mass, visceral fat mass, IMGD, ED_{50} insulin for stimulation of IMGD and ED_{50} for suppression of EGP and lipolysis will be determined from the euglycemic clamp study (57;101;117). Flux data will be examined both per kg fat-free mass (FFM) and per kg total fat mass. SI will be determined by minimal model analysis of the FSIGT glucose and insulin values (120;121). The disposition index will be determined as the product of (1) SI and AIRg from the FSIGT and (2) of the insulin secretion rate from Study 2 and IMGD (121-124).

e. Anticipated Results/Potential Limitations/Alternative Approaches/Future Studies. These studies will provide a comprehensive overview of insulin action in A8(+) and A8(-) PCOS women. We anticipate that insulin sensitivity will be similar in A8(+) and A8(-) individuals and that both groups of PCOS women will be more insulin resistant than control women. If we find that both A8(+) and A(-) PCOS have similar degrees of insulin resistance compared to control women, this will suggest that there are additional genes that produce insulin resistance in PCOS. We will genotype all control subjects for A8 but will not exclude A8(+) subjects. If A8 is associated with a metabolic phenotype in the general population, it will be more difficult to detect differences in PCOS subjects compared to control populations with A8(+) subjects. However, we have been able to detect significant differences in PCOS women, and their male as well as female first-degree relatives compared to control subjects without stratifying the control population on the basis of A8 (Preliminary Studies). We are measuring insulin action with the "gold standard" method the euglycemic clamp (112;125). We will also measure insulin sensitivity with an FSIGT (125).

We are employing a matched study design of A8(+) compared to A8(-) PCOS women to give us the maximum statistical power. We are also including a control group to assess the impact of PCOS, in addition to A8 status on the parameters under study. Both groups of PCOS women will be matched based on BMI, total adiposity and visceral adiposity, age, and ethnicity (see Subjects Section D4 below). In Aims 1 and 2 we will not investigate the independent impact of obesity. We anticipate that the majority of our PCOS subjects will be obese. If we find that there are metabolic differences related to A8, in future studies we will investigate the independent impact of obesity.

While the possibility exists, it is highly unlikely that the signal at the marker locus D19S884 is a false positive result since we have replicated the association in a second sample of families, the association has also been replicated in an independent case-control study, and there is linkage at the marker locus by affected sib pair analysis (Project 2). The marker locus is also associated with a metabolic phenotype. This evidence is already much stronger to support the presence of a PCOS susceptibility gene in this region than the reported associations of variants in many other candidate genes that have not been detected by linkage analysis (11;26;27). The fact that we can detect this region in linkage analysis in a relatively small sample of affected sib pairs suggests the locus contains a susceptibility gene with a large effect (84). The identification of this same marker in the TDT analysis suggests that the marker is very near to the susceptibility locus because linkage disequilibrium is maintained over relatively small genetic distances (9;126).

D2. Aim 2. To test the hypothesis that A8(+) PCOS women have more profound defects in insulin secretion compared to A8(-) PCOS women.

a. Rationale. Our preliminary studies suggest that A8 is associated with decreased insulin secretion since 2 h post-challenge glucose levels are significantly higher whereas insulin levels are not increased in A8(+) PCOS women. Consistent with this hypothesis, insulin levels are decreased and glucose levels increased in A8(+) PCOS women during FSIGTs. These changes most likely reflect decreased β-cell responses to glucose and/or sulfonylurea (since there were decreased insulin responses after IV tolbutamide during the FSIGT, Preliminary Studies) (91;111;127). The mechanism for this abnormality could be either decreased sensitivity to glucose as is found in MODY due to glucokinase mutations or decreased maximal responsiveness, suggesting diminished β-cell mass as is seen in DM2 (111;127). Decreased responses to tolbutamide could also be secondary to a defect in the sulfonylurea receptor (Project 4) (91). It remains possible that these changes in insulin levels reflect abnormalities in insulin clearance or in an incretin (120).

b. Experimental Approach. No consensus has been reached on the best method to assess islet function (128-130). Accordingly, we will utilize well-validated methods that have been employed to delineate islet cell function in conditions such as DM2, IGT and MODY (111;130-132). Because we will perform several tests of islet function as well as tests of insulin action (Aim 1) in the same subjects, blood volume requirements must also be considered in selecting these tests. Insulin responses to oral glucose and to a mixed meal will be assessed to investigate whether there are defects to suggest the possibility of decreased secretion of an incretin, such as glucagon-like peptide-1 (GLP-1) (133;134). GLP-1 levels will be measured during these oral challenges using an assay that measures the active form of this rapidly degraded hormone. An actual mixed meal will be given rather than a liquid meal to better recreate the physiologic situation (135;136). A graded glucose infusion will be performed to examine the dose-response features of insulin secretion. This test will allow the assessment of β-cell sensitivity to glucose (111;137;138). Insulin secretion rates will be determined during the graded glucose infusion by deconvolution of C-peptide kinetics. It is no longer possible to directly measure C-peptide clearance in humans because recombinant human C-peptide is no longer manufactured. Fortunately, the population-derived standard C-peptide clearance parameters for subjects of similar age, sex, and body surface area have previously been validated in PCOS women by direct measurement of C-peptide clearance by Polonsky and Ehrmann (139-141). These parameters will be used to derive insulin secretion rates.

To assess maximal β-cell secretory capacity as well as α-cell function, we will perform a glucose-dependent arginine stimulation test as modified by Larsson and Ahren (127;130). A maximally stimulating dose of arginine (5 g) will be administered at baseline and at 13-15 mmol/L glucose. After a 2.5 h washout necessary to prevent a priming effect of prior hyperglycemia, arginine will be administered again at >25 mmol/L glucose (maximally stimulating glucose for insulin release) (127). This test measures glucose potentiation of insulin secretion and glucose inhibition of glucagon secretion as well as basal and maximal β- and α-cell secretion (130). Subtle defects in proinsulin processing can also be unmasked with this test (142). The blood volume requirements for this test are less than for arginine-hyperglycemic clamp protocols, which yield similar information (127;143). Defects in arginine-stimulated glucagon as well as insulin secretion have been found in MODY due to mutations in HNF-4α and in postmenopausal women at risk for glucose intolerance (111;144).

We will also perform an FSIGT with tolbutamide to assess acute insulin responses to intravenous glucose and insulin responses to tolbutamide since our preliminary studies show that the latter are markedly decreased in A8(+) PCOS (Preliminary Studies) (120). This test also permits the assessment of SI and the disposition index (145). We will compare the FSIGT to more complex tests of islet function to determine whether the FSIGT can be used alone in future studies. We will relate insulin secretion rates to insulin sensitivity determined by euglycemic glucose clamp (the disposition index) in Study 1 (132;146). All subjects will have normal glucose tolerance to remove the confounding effects of hyperglycemia on the study outcomes.

c. Protocol Study 2

i. Subjects. Age- and weight-matched A8(+) and A8(-) PCOS and control women

ii. OGTT. Performed as part of scr[redacted]ng visit 1 (see below)

iii. Visit 1. Meal tolerance test with 500-calorie test meal consisting of 55% carbohydrate, 15% protein, 30% fat

iii. Visit 2. Graded glucose infusion

iv. Visit 3. Glucose-dependent arginine stimulation test

v. Visit 4. FSIGT with tolbutamide

d. Endpoints. The endpoints for the OGTT and meal tolerance test will be glucose, insulin and GLP-1 levels and area-under-the-curve (AUC) (133;135;136). Insulin secretion rates will be the endpoint in the graded glucose infusion (147). Insulin secretion and clearance rates will be measured by deconvolution of C-peptide kinetics using population-derived parameters (139;140;148). Acute insulin (AIR), C-peptide (ACR), proinsulin (APR) and glucagon responses (AGR) will be determined at each glucose level during the glucose-dependent arginine stimulation test. The slope of these parameters between baseline and 14 mmol/L glucose will be used to assess glucose potentiation of insulin secretion and glucose inhibition of glucagon secretion (130;142). The proinsulin:insulin ratio will be determined as a measure of proinsulin processing (142). Acute insulin response to glucose (AIRg) will be determined as AUC insulin levels from 2-10 min, and insulin responses to tolbutamide will be AUC insulin levels 22-180 min of the FSIGT (120;126;149).

e. Anticipated Results/Potential Limitations/Alternative Approaches/Future Studies. These studies will provide a comprehensive overview of insulin secretion in A8(+) and A8(-) PCOS women. We anticipate that A8(+) PCOS women will have decreased glucose and tolbutamide-stimulated insulin secretion compared to A8(-) PCOS women. We expect that PCOS women will have defects in insulin secretion when compared to control women, independent of obesity, consistent with our previous studies. Potential differences in oral glucose or meal-stimulated, compared to intravenous glucose-stimulated insulin secretion, would suggest differences in the secretion of an incretin (133;150). The only incretin that we will measure is GLP-1 (133). *In utero* testosterone exposure causes defects in insulin secretion in female rhesus monkeys, and this effect will be explored further in Dr. Abbott's Project (Project 3). Dr. Levine (Project 4) has preliminary evidence to suggest that androgens decrease the activity of K^+_{ATP} channels. Sulfonylureas stimulate insulin secretion through activation of one of these K^+_{ATP} channels, known as the sulfonylurea receptor (91). Since androgens can decrease the function of these channels (Project 4), a decrease in sulfonylurea-stimulated insulin secretion could reflect androgen programming. This finding would suggest that A8(+) PCOS were exposed to higher androgen levels during development. This hypothesis will be directly tested by measuring amniotic fluid androgen levels in A8(+) and A8(-) female PCOS offspring in Aim 3 of this Project.

There are a variety of methods to assess insulin secretion; no "gold standard" exists (128;130;132;137;149;151). The methods that we have selected have been used successfully to characterize the different defects in islet cell function in the various types of MODY (111). Hyperglycemic clamp studies and studies of pulsatile insulin secretion would be of interest but require large volumes of blood (152-155). These studies can be considered in the future if secretory defects are detected in the initial series of islet tests. Further, if a consensus develops on the best method for assessing β-cell function, we will certainly employ it.

D3. Aim 3. To test the hypothesis that there is *in utero* testosterone excess, altered insulin secretion and/or intrauterine growth retardation in the female offspring of PCOS women. To determine whether A8(+) female offspring have more profound changes in these parameters compared to A8(-) female offspring.

a. Rationale. A key component of the hypothesis of this SCOR is that there is intrauterine androgen excess in PCOS. To test this hypothesis, we will determine whether there are increased levels of T or other androgens in the amniotic fluid in PCOS compared to control pregnancies. Steroids in all the delta 4 and delta 5 pathways of androgen and estrogen biosynthesis are measurable in amniotic fluid from pregnancies with normal male and female

fetuses. It is possible to detect significant sex differences in T and in estradiol levels in amniotic fluid from both mid and late gestation as well as in cord blood (156-163). Further, significantly elevated levels have been found in late gestation amniotic fluid from diabetic mothers with female fetuses (163). This finding indicates that it is not only possible to measure amniotic fluid T levels but also to find significant increases of T in amniotic fluid from female fetuses in certain circumstances (163). Dehydroepiandrosterone (DHEA) and its sulfate are the major products of the fetal adrenal (164;165). The sex differences in fetal gonadal steroids indicate that the fetal ovary as well as the fetal testis are active (156;158;159,163). Ovarian theca-lutein hyperplasia has been found in female infants of diabetic mothers (163). Human chorionic gonadotropin and insulin can be elevated in diabetic pregnancies, and both of these hormones can act in synergy to stimulate ovarian androgen production (163;166). The putative genetic variation resulting in hyperandrogenemia in adult PCOS women could increase fetal ovarian and/or adrenal steroidogenesis in affected offspring (6).

The fetal pancreatic β-cell function can be assessed by measuring amniotic fluid insulin levels, and these levels are measurable in mid gestation at the time of amniocentesis for genetic testing (167). Amniotic fluid insulin is derived from renal clearance of insulin and reflects integrated fetal insulin output (168). Elevated amniotic fluid insulin levels predict the development of maternal gestational diabetes (169). According to our hypothesis, there should be decreased fetal insulin secretion because of an androgen-mediated decrease in K^+_{ATP} channel activity. Decreased fetal insulin secretion could result in decreased fetal growth and IUGR according to the "fetal origins" or "Barker" hypothesis (68;71;170). IUGR is associated with the insulin resistance syndrome and, possibly, PCOS in adult life (171;172). Ibanez and colleagues have found an increased prevalence of low birth weight in girls with PCOS (80). Birth weights of prenatally androgenized monkeys are significantly lower than those of control animals (Project 3).

Clearly, if PCOS has a genetic component, not all female offspring will be affected. In reproductive-age female relatives we can determine affected status based on androgen levels (Preliminary Studies) (6). However, in other individuals the phenotype is unknown – e.g. premenarchal girls, postmenopausal women and males. Our preliminary studies in the brothers of PCOS probands suggest that A8 may be able to identify an "affected" subgroup since A8(+) males have evidence for a metabolic phenotype (Preliminary Studies). Accordingly, we will use A8 status to identify "affected" female offspring. We have designed this study so that we will have an adequate sample of A8(+) and A8(-) female offspring to determine whether any of the parameters under study differ.

An effect of genotype of the offspring on birth weight has been reported in PCOS in one study where the magnitude of low birth weight was associated with certain alleles of the insulin gene VNTR (173). We will also be able to examine the impact of maternal and fetal A8 because only 50% of the offspring of heterozygous A8(+) PCOS mothers will be A8(+). The impact of maternal and fetal glucokinase gene genotype on birth weight was examined in MODY families and this study suggested that decreased fetal insulin secretion in glucokinase gene mutation positive fetuses resulted in low birth weight whereas this mutation in the mother with a normal fetus resulted in higher birth weights, an outcome that is perhaps related to maternal glycemia (174).

b. Experimental Approach. We will prospectively examine amniotic fluid and cord blood androgen and insulin levels in PCOS pregnancies with A8(+) and A8(-) female offspring. To determine the effect of PCOS, we will include age-, weight-, parity-, and ethnicity-matched pregnant control women. All pregnant women will be undergoing clinically indicated week 15-19 amniocentesis for genetic testing. This time in human gestation corresponds to day 40-80 in rhesus gestation when testosterone will be administered to the monkeys in Project 3 (165). Accordingly, this time will be ideal to sample amniotic fluid to test the hypothesis that androgens are elevated at an analogous time in PCOS pregnancies to the time of their administration to monkeys in order to produce the prenatally androgenized animals (Project 3).

We will measure the androgens, T, androstenedione, and DHEA, as well as DHEAS, which is produced primarily by the adrenal (164;165). Insulin and C-peptide levels will also be measured in amniotic fluid samples and cord blood. Cord blood will be obtained only from uncomplicated vaginal deliveries or elective caesarean section. It has been possible to determine sex differences in gonadal steroid levels in such samples (160). Cortisol levels will be measured to provide a gross index of fetal stress, which could also increase adrenal androgen levels. However, these levels are similar in vaginal deliveries to those in elective caesarean sections (175). Thus, as long as the vaginal

delivery is uncomplicated, it does appear more stressful to the fetus than can an section (175;176). Birth weight and anthropometric measures will determine gestational age. PCOS offspring will be compared in these parameters not only to control but also to the population of infants delivered at Prentice Women's Hospital during the same time period. Prentice Women's Hospital is part of the Maternal Fetal Medicine Network and Dr. Wang, Co-Investigator, is also involved in that study. Thus, she has access to their database. In addition, our Co-Investigator Boyd Metzger is Principal Investigator on the Hyperglycemia and Pregnancy Outcomes (HAPO) Study and is establishing normative data for all of the anthropometric measurements we plan to use in a large population of infants at Prentice and nationwide (see below).

In infants of diabetic mothers, fetal amniotic fluid insulin levels are strongly correlated with the magnitude of the weight of the fetus ascribed to excess adiposity (168;177). We will express birth weight either as a percentile rank relative to that expected for a reference population adjusted for gestational age or as relative weight in proportion to relative length of a reference population. These methods to calculate symmetry or ponderal indices define neonatal anthropometrics much more accurately than birth weight at arbitrary levels (178;179). The volume/mass of subcutaneous fat is known to correlate with body composition. Measurements of skinfolds will be used to derive an index of body fat in newborns (180). Recently, Catalano et al (179) demonstrated very good correlation between carefully standardized measurements of flank skinfold thickness and more direct indices of body fat in newborn infants of Caucasian, African-American and Hispanic mothers.

c. Protocol Study 3

i. **a. Subjects.** Pregnant PCOS and age-, weight-, parity- and ethnicity-matched control women undergoing indicated amniocentesis.

ii. **Clinically Indicated Amniocentesis.** Amniotic fluid (5 ml) will be collected at the time of an indicated week15-17 amniocentesis for genetic testing. A maternal blood sample will be obtained for DNA.

iii. **Delivery.** After delivery, vaginal or elective caesarean section, the umbilical cord will be double clamped, and cord blood will be obtained. The placentas will be weighed and frozen for genetic analyses.

iv. **Anthropomorphic Measurements.** Birth weight, anthropomorphic measurements and skin folds will be determined in the newborns.

v. **Endpoints.** Amniotic and cord blood levels of T, DHEA, DHEAS, androstenedione, insulin and C-peptide. Ponderal index (birth weight/crown-heel length^3 x 100); subscapular and triceps skin fold thickness, circumference of head, chest, abdomen and upper arm; and placental weight.

e. Anticipated Results/Potential Limitations/Alternative Approaches/Future Studies. Based on our preliminary studies showing decreased insulin secretion in A8(+) PCOS women, it is our hypothesis that insulin levels will be lower in A8(+) female fetuses/offspring. We propose that the mechanism for lower insulin levels is prenatal androgen excess; hence, we also expect to find that androgen levels are higher in amniotic fluid and/or cord blood of A8(+) than of A8(-) female offspring. Finally, we expect that the A8(+) offspring will be smaller for gestational age because of reduced insulin levels (174;181;182).

Only 50% of the offspring will receive the maternal A8 allele if the mother is heterozygous. Further, since A8 is a common allele in the population (~20%), it is possible that the offspring may receive a paternal A8 allele. Accordingly, there are several possible outcomes if the A8-linked gene has an impact on the study endpoints. It is our prediction that only A8(+) female offspring (regardless of which parent contributed A8(+) allele) will be affected because the putative genetic variant directly affects fetal steroidogenesis (our hypothesis) and/or metabolic parameters (also possible). In addition, an A8(+) PCOS mother may have an impact on the offspring's phenotype. If this is the case we should see an effect in both A8(+) as well as A8(-) female offspring of A8(+) mothers. We may see an enhanced effect if both PCOS mother and female offspring are A8(+). In MODY due to glucokinase gene mutations, an impact of both maternal and fetal genotype on birth weight has been found (174). Since our study is

designed to detect changes related to [redacted] status in the offspring, we may not have a[redacted]ate statistical power to detect changes in these subpopulations (e.g. [redacted]8(+) mother, A8(-) offspring). If we have evidence for such effects, we will furt[redacted] investigate this in future studies. Finally, it is possible that non-genetic factors related to PCOS status have an effect. For example, it is possible that maternal hyperandrogenism and/or insulin resistance has a negative impact on placental development. Indeed, an alternate explanation for changes observed in the prenatally androgenized monkeys (Project 3) is that the induction of maternal androgen excess alters placental function resulting in the observed phenotype. The presence of differences in placental weights in PCOS compared to control women would be consistent with this possibility (68). Further, the presence of changes in any of the endpoints in the majority of PCOS offspring independent of A8 status would suggest that there is an effect of maternal PCOS.

According to our hypothesis, androgen levels will be higher and insulin levels lower in the A8(+) female offspring of PCOS than in A8(-) PCOS or in control female offspring. However, we have shown that PCOS women are insulin resistant independent of A8 status (Preliminary Studies), suggesting that there are additional genes modulating insulin action. Thus, it is possible that we will find increased insulin levels in PCOS offspring because of genetically-determined insulin resistance. Primary fetal insulin resistance is another cause of IUGR best exemplified by Leprechaunism, but there is evidence to suggest that other genetic variants that produce insulin resistance are associated with decreased gestational size(183;184). It is also possible that we will fail to detect changes in androgen or insulin levels because such changes are subtle. In humans, there is no alternative approach to address this question.

Our interpretation will be that elevated amniotic fluid androgen levels reflect increased androgen secretion by the fetal adrenal and/or ovary rather than maternal androgens. Endogenously produced maternal androgens are thought to be completely aromatized into estrogens by the placenta (185). Only in instances of profound maternal androgen excess is the aromatase capacity of the placenta exceeded resulting in virilization of a female fetus (185). However, it is possible that there is an increase in androgens in female offspring of PCOS mothers due to maternal androgen excess. The cord blood samples, which reflect the fetal circulation, will address this issue.

Clearly, not all infants who are small for gestational age have IUGR. Size at birth is influenced by many genetic, environmental and nutritional factors. Any population is expected to include a proportion of infants that are smaller (SGA) or larger (LGA) than expected for the gestational age. Indeed, the commonly used standards place 10% of the population in each of these categories. We expect to see some well-proportioned newborns with low and high birth weights. Dr. Metzger's (Co-I) HAPO Study has total sample size of ~25,000 infants. The relatively large number of participants enrolled for the HAPO Study at multiple US sites, including Prentice Women's Hospital (1500 or 1750 per site), will permit the calculation of gestational age specific distributions of total body weight, length, chest, abdominal and head circumferences, and skin folds. We will use these data as normative data for this study, in addition to data that will be obtained in the concurrently studied control female offspring. We will also adjust for maternal factors such as BMI, weight gain during pregnancy, history of prior small for gestational age infant, maternal birth weight, parity and tobacco use in our data analyses. Our study has the advantage of prospectively evaluating gestational age. Other studies have been retrospective and are subject to a number of confounding factors (80;172).

Approximately 50% of the women we recruit for this study will have male offspring. We will determine the sex of the fetus before performing hormone assays or anthropometric measurements because of budget constraints. However, we will collect birth weight data to determine prospectively whether there is an effect of PCOS on male offspring. If the current study finds changes in female offspring, in future studies we will investigate these endpoints in male offspring.

D4. Subjects

a. General Selection Criteria for Subjects. All subjects will be in good health with no chronic diseases. No subjects will smoke. No subjects will have hypertension. PCOS and control women will be between 18-40 years old. No subjects will be taking any medication known to affect reproductive hormone levels or carbohydrate metabolism for at least one month prior to the study, except for oral contraceptive agents, which will be stopped at

Demographics of Anticipated Study Population-Gender and Minorities

	American Indian or Alaska Native	Asian	Native Hawaiian or other Pacific Islander	Black or African American	Hispanic or Latino	White	Other or Unknown	*Total*
Female	0	0	0	0	0	580	0	*580*
Male	0	0	0	0	0		0	
Unknown	–	–		–	–	–	–	–
Total	0	0	0	0	0	580	0	*580*

least three months prior to the study. No subject will engage in 3 or more 20 min sessions of aerobic exercise, and subjects will refrain from all exercise for 3 d prior to each study.

b. Selection Criteria for PCOS. Only PCOS women with biochemically documented hyperandrogenemia (T and/or uT ≥ 2 standard deviations above the mean for normal ovulatory control women) and chronic anovulation (≤6 menses/y) will be studied in order to fulfill the strictest and the least controversial diagnostic criteria for PCOS. We have used these criteria for all of our studies of PCOS (29). Androgen-secreting tumors, Cushing's syndrome, and hyperprolactinemia will be excluded by appropriate tests in all PCOS women. In Studies 1 and 2, A8(+) and A8(-) PCOS will be matched for BMI and body composition.

c. Selection Criteria for Control Subjects. To exclude disorders associated with insulin resistance, control subjects will have no personal history of hypertension or hypertriglyceridemia and no first-degree relative with DM2 (92;186). Control women will have regular menses every 27-35 days, no hirsutism (Ferriman and Gallwey score <8), and normal T, uT, and DHEAS levels.

d. Pregnant Women. Pregnant women will have no history of gestational diabetes mellitus, eclampsia, pre-eclampsia or any medical disorders complicating their pregnancies. They will be undergoing amniocentesis for genetic screening. Pregnant PCOS women will have been diagnosed with PCOS using the criteria outlined above when possible. If the diagnosis was not confirmed before pregnancy, it will be confirmed after pregnancy. The hormonal evaluation after pregnancy will be performed at least 3 months after nursing has ceased completely to avoid the confounding effects of hyperprolactinemia. PCOS women will have non-classic congenital adrenal hyperplasia excluded by an ACTH test (see below)(187). Genetic testing will be performed if there is any doubt regarding this diagnosis since adrenal hyperplasia in an offspring would result in fetal adrenal androgen excess and confound Study 3. Pregnant control women will have a history of completely regular menstrual cycles. Pregnant control women will have no history of hirsutism and will have no clinically significant hirsutism (Ferriman and Gallwey score<8) or alopecia on physical examination. Control women will have a blood sample obtained 3-6 mons after they have stopped nursing and resumed regular menses to ensure that they fulfill hormonal criteria outlined above for control women. Any pregnant woman who develops gestational diabetes will be excluded from the analysis.

e. Gender, Children, and Minorities Inclusion. There are racial and ethnic differences in insulin action, body fat distribution, lipolysis and birth weight (81;117;188). Thus, an additional complete sample of subjects in each of these groups would be required for adequate statistical power. As the sample sizes needed to address the Aims of this proposal are large, we will first study Non-Hispanic White women. In future studies, we will investigate the impact of ethnicity. Newborns, women ages 18-21 years defined as children by NIH, as well as pregnant women will be studied. Men will not be study at this point because the known PCOS phenotype occurs in women. In future studies, we will study men if we find that A8 is associated with a metabolic phenotype in PCOS women.

D5. Screening

a. Screening Visit 1 Non-pregnant Women. All subjects will have a history, physical examination, blood drawn for T, uT, DHEAS, prolactin, TSH and DNA. They will have 75 g glucose OGTT in the post-absorptive state after a

3 d 300 g carbohydrate preparatory diet and an overnight fast. Blood for glucose and insulin levels will be obtained at baseline and every 30 min for 3 after the oral glucose load. All subjects will have normal glucose tolerance according to WHO criteria (189). Potential PCOS subjects will have a 1 h ACTH stimulation test with baseline and 1 h 17-hydroxyprogesterone levels to exclude non-classic congenital adrenal hyperplasia (190). All PCOS women who fulfill diagnostic criteria will be genotyped and have body composition studies outlined below to address Aim 1. Since the prevalence of glucose intolerance is ~40% in obese PCOS women, and the prevalence of homozygous and heterozygous A8(+) PCOS is ~30% of PCOS women (allele frequency of ~18% in our studies and ~20% in CEPH families, Project 2), we will need to screen substantially more PCOS women to enroll adequate numbers with normal glucose tolerance and who are A8(+) (191;192)(Table 4).

b. Screening Visit 2 Non-Pregnant Women. All subjects who fulfill the study subject criteria will have body composition determined by dual photon x-ray absorptiometry (DEXA) and visceral fat determined by computerized tomography (CT) scan (52;193;194). Reproductively-normal women will be matched for VAT determined by DEXA and CT scans. We will also attempt to match PCOS and control women for total fat. This may not be possible in the obese group since we have previously found that obese control women matched to PCOS women for fat mass have lower WHR and SQ adipocyte size, suggesting they have less VAT (57). Thus, it is possible that the VAT-matched control women will be more obese than the PCOS women. However, Dumesic and colleagues were able to match obese PCOS and control women for total fat and VAT (52). We will also determine whether significant differences exist in total or visceral fat mass in A8(+) compared to A8(-) PCOS matched for BMI (114).

c. Study Preparation and Timing. In non-pregnant women, studies will be performed in the morning after a 10 h overnight fast and a 3 d 300 g carbohydrate preparatory diet. A bedtime snack will be given to ensure that the duration of the overnight fast is 10 h. All studies will be performed in the follicular phase of the menstrual cycle in control women and at a time of documented anovulation by serum progesterone levels <2 ng/mL in PCOS women. All studied will be separated by at least 24 h, except for euglycemic clamp studies, which will be separated by at least 7 d. Because of the potential deleterious effects of prolonged fasting in pregnant women, the amniocentesis will be performed in the non-fasting state. Further, fetal amniotic fluid insulin levels are not acutely altered by maternal food intake (195).

d. Data Analysis, Sample Size Estimates. A8(+) vs A8(-) PCOS women will be compared to control women by 1-way ANOVA. The subjects will be matched for age and BMI. The endpoints in the A8(+) and A8(-) PCOS and control offspring for amniotic fluid and cord blood hormone levels and for anthropometric data at birth will also be compared by 1-way ANOVA. Anthropometric measurements will also be compared to the concurrently obtained normative data, and the number of A8(+) and A8(-)PCOS and control offspring above and below the 10% for gestational age will also be compared. There will be 3 contrasts of interest: A8(+) PCOS vs A8(-) PCOS, A8(+) PCOS vs control, A8(-) PCOS vs control. To obtain a conservative estimate of sample size, we have used the Bonferroni adjustment for multiple comparisons to adjust the level of α for the number of comparisons. Thus for 3 comparisons α=0.017. Since we will perform transformations to achieve homogeneity of variance where appropriate, sample sizes have been calculated for unpaired *t*-tests assuming equal variance with 80% power. The same sample of PCOS women will participate in Studies 1 and 2. The sample size estimate of 15-30 subjects per group is based on differences in AUC insulin and AUC insulin: glucose during FSIGTS (Preliminary Studies) in obese A8(+) PCOS compared to obese A8(-) PCOS women. We will enroll 35 subjects per group because of anticipated drop-out and technical problems. We will study only subjects with normal glucose tolerance to remove the potential confounding effects of hyperglycemia on the study outcomes. Since the prevalence of IGT and DM2 is ~40% in obese PCOS women, we anticipate that many PCOS women who

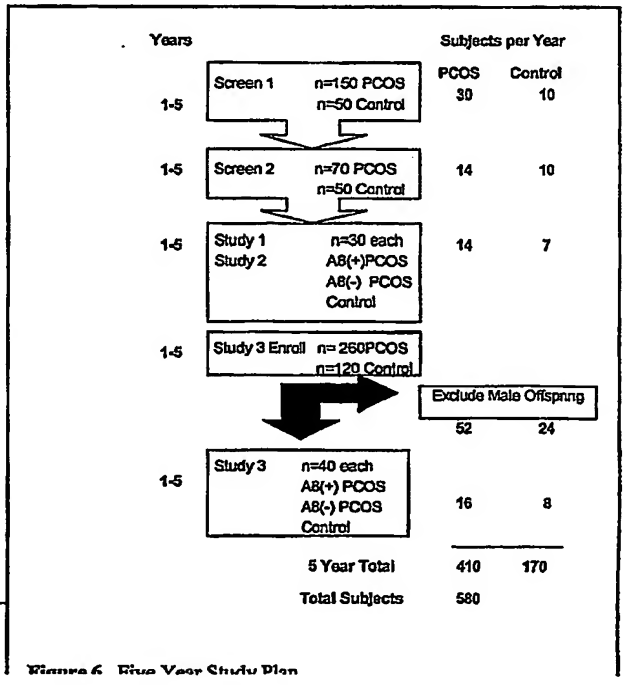


Figure 6. Five Year Study Plan

fulfill our other screening criteria w[illegible]e excluded (191;192). We will include th[illegible]women in an analysis of the prevalence of IGT and DM2 by WH[illegible]iteria in A8(+) compared to A8(-) PCOS w[illegible]en using the OGTT data from the [illegible]ening study.

The sample size estimate for Study 3 is based on the observed difference in amniotic T levels in female fetuses of diabetic mothers compared to control and in a 25% decrease in mid gestation amniotic fluid insulin levels(163;196-198). For an α=0.017 with a power of 80%, 40 subjects will be required per group. This sample size will be adequate to detect a 25% difference in late amniotic DHEAS and androstenedione levels as well as a 10% decrease in birth weight (172). In order to obtain 40 each A8(+) and A8(-) female offspring of PCOS, it will be necessary to enroll ~240 pregnant PCOS since only ~50% of conceptions will be female (n=120) and ~33% will be A8(+) (n=40) and ~66% A8(-) (n=80) (Figure 6). We will enroll ~260 pregnant PCOS because of anticipated dropouts, disqualifications for medical problems during pregnancy (e.g. gestational diabetes) and technical difficulties (Table 4). We will enroll ~120 pregnant control (for n=60 female offspring) women of comparable age-, weight and ethnicity.

b. Feasibility. There are ~8,000 deliveries in 2000-2001 and ~900 genetic amniocenteses per year at Prentice Women's Hospital of Northwestern Memorial Hospital. The Division of Reproductive Endocrinology estimates that they have ~50 PCOS pregnancies per year and those women usual deliver at Prentice Hospital. The private physician staff in Obstetrics and Gynecology has a large population of pregnant PCOS from ovulation induction with clomiphene citrate. Dr. R. Barnes (Co-I) will assist in the recruitment of pregnant PCOS women at Prentice Women's Hospital. In order to ensure that there is an adequate sample of pregnant PCOS women, Dr. Richard Legro from Hershey-Penn State Medical Center will be a consultant. He is a longtime collaborator of the PI and is a Co-Investigator on the PI's grant to study PCOS families (HD 34449, Project 1). Accordingly, he is already recruiting and phenotyping PCOS women by the same methods as outlined in this application. Many of the PCOS women are part of his infertility practice . Dr. Legro estimates that he has ~50 PCOS pregnancies per year in his practice. Dr. Legro's Study Coordinator for the PCOS Family study will collect cord blood samples and perform the anthropometric measurements on the newborns on a fee-for-service basis. With these two sources of pregnant PCOS women, it will be possible to recruit ~260 pregnant PCOS women in the five year duration of the award. The PI typically studies 100 PCOS women per year and will have no difficulty recruiting non-pregnant PCOS women for this study. Dr. David Ehrmann from the University of Chicago has agreed to be a consultant and to share his population of PCOS women with the PI (see letter).

c. Study Plan (Figure 6). All studies will run concurrently. Approximately 40 (30 PCOS, because of the high exclusion rate for glucose intolerance, and 10 control) subjects will be screened per year to enroll 21 per year in Studies 1 and 2. Approximately 50 pregnant PCOS and 15 pregnant controls will be enrolled per year. Amniotic fluid samples will be obtained and the sex of the offspring determined. Only female offspring will be genotyped and have sampling for cord blood and anthropometric measurements.

D7. Methods

a. Assay Procedures Adult Blood Samples. All blood samples (except for glucose clamp glucose samples, which are assayed at the bedside) will be collected in 4 C tubes and will be kept on ice. The tubes will be centrifuged at 4 C within 30 min. Plasma and sera will be stored at -80 C until analysis. In samples from adult women, glucose, insulin, proinsulin, T, uT, DHEAS, prolactin, TSH, progesterone cortisol and 17-hydroxyprogesterone levels will be determined by our previously reported techniques (6;57;199). These assays all have <10% interassay coefficients of variation. Glucagon and C-peptide will be collected in 4 C EDTA tubes with aprotinin (500 KIU/mL, Bayer, West Haven, CT) and assays will be performed in the GCRC Core Laboratory with kits from Linco Research, Inc (St Charles, MO). Blood for GLP-1 will be drawn in 4 C EDTA tubes containing DPP-IV inhibitor (10 µL/mL, Linco Research, Inc). GLP-1 will be extracted with 95% ethyl alcohol and levels of the biologically active form [GLP-1(7-37) or GLP-1(7-36) amide] determined by radioimmunoassay with reagents from Linco Research, Inc (200). The guinea pig-anti-GLP-1(7-36)amide antibody is directed to the N-terminus of active GLP-1. The specificity of this antibody is human GLP-1(7-36)amide 100%, human GLP-1(7-37)amide 100%, human GLP-1(9-36) <1%, human GLP-2 not detectable, human glucagon 16%, human insulin not detectable, human GIP not detectable, human VIP not detectable.. The standard and tracer are prepared with GLP-1(7-36) amide (Linco Research). The recovery of

standard after alcohol extraction is 3[redacted]8%. Blood for FFA will be drawn in 4 C t[redacted] containing 0.1% EDTA and paraoxon (0.4 mg/mL) and assayed [redacted]g a kit from Wako (NEFA C, Wako Pure [redacted]emical Industries, Richmond, VA[redacted]01).

b. Assays Amniotic Fluid and Cord Blood. In order to detect insulin levels in mid gestation amniotic fluid, we will use the Access Immnuoassay system and Ultrasensitive insulin assay kit (Beckman Coulter, Brea, CA) with a sensitivity of 0.03 μU/ml. These reagents have been successfully used to measure mid amniotic fluid insulin levels (198), which ranged from 0.2-7.4μU/ml. These assays will be performed in the Northwestern Memorial Hospital Clinical Pathology Laboratory (see letter). Extraction and Celite chromatography will be used to measure T, androstenedione, DHEA levels in amniotic fluid and cord blood with methods previously as reported (158;159;163,202). These assays will be performed by Esoterix Center for Clinical Trials (formerly Endocrine Sciences, Calabasas Hills, CA) (see letter). The sensitivity of the assays are T 0.03 ng/ml, androstenedione 0.01 ng/ml, and DHEA 0.01 ng/ml. Using mass spectrometry, the ranges for female fetus mid gestation amniotic levels of these androgens are: T (0-0.27 ng/ml), androstenedione (0-2.71 ng/ml), and DHEA (0.19-1.77 ng/ml)(161). C-peptide and DHEAS levels will be measured as outlined above.

c. DNA Isolation and Genotyping D19S884. Fetal DNA will be obtained from cell cultures established for genetic analysis at the time of amniocentesis. Maternal DNA will be isolated from a blood sample. DNA will be extracted as reported (7). For each individual genotyped, 45 ng of DNA will be amplified in a total volume of 8 μl in the presence of 200 μM dNTPs (Amersham Pharmacia Biotech), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM MgCl2, 0.36 units AmpliTaq polymerase (Roche Molecular Systems, Branchburg, NJ), and 0.5 μM of each primer. The forward primer is fluorescently labeled with Tet and electrophoresed in the presence of an internal size standard (Genescan 500) on 4% acrylamide, 5M urea denaturing gels using a 377 DNA sequencer (PE Applied Biosystems, Foster City, CA). Genotypes will be determined using the GeneScan Analysis and Genotyper programs (PE Applied Biosystems, Foster City, CA).

d. Body Composition and Visceral Fat. Visceral adipose mass will be determined using a single CT slice at the L2-3 vertebral interspace (52;194). The product of the percent visceral adipose area in that slice and DEXA determined total abdominal fat mass will be used to estimate total abdominal visceral adipose mass. Total body composition will be determined using DEXA (193).

e. Assessment of Gestational Age, Body Fat and Anthropometrics. The birth weight will be recorded on an electronic scale to the nearest gram. Recumbent crown-heel lengths to the nearest mm will be recorded using a length board. Using a standard tape measure, the following measurements will be taken: head circumference (frontooccipital), chest circumference at the level of the nipples, umbilical abdominal circumference (just above the umbilicus), and mid-upper arm circumference. Using calipers (Lange calipers, Beta Technology Inc, Santa Cruz, CA), both subscapular and triceps skinfold thickness will be obtained in duplicate, on the left side of the body. All measurements will be carried out by a maximum of three observers trained identically to obtain measurements with periodic assessments of inter- and intra-observer variation. In prior studies, the coefficient of variation for the duplicate skinfold measurements ranged from 5-6% to 1-2% over time (179;203). Drs. Wang and Metzger will assist in the oversight of these measurements. Dr. Metzger has extensive experience standardizing these measurements at multiple sites for his HAPO study. He has developed a Method of Operations and forms (appendix) that we will use.

f. Glucose Clamp Studies. Euglycemic glucose clamp studies will be performed as we have previously reported (31;57;58). A pancreatic clamp will be performed with SRIF, 140 ng/kg FFM/min (Bachem Bioscience, Inc., King of Prussia, PA), and GH, 6 ng/kg FFM/min (Genentech, Inc., South San Francisco, CA), starting at -5 min (117). Basal EGP and its suppression by insulin will be assessed using a primed, continuous infusion of [6,6-^{2}H]- glucose 2 mg/m^2/min (Isotec, Miamisburg, OH) starting at -120 min (57;115). Sequential primed insulin doses of 2.5-400 mU/m^2/min will be administered for 2 h at each dose with euglycemia maintained with a variable infusion of 20% glucose enriched to ~2.5% with [^{2}H]-glucose (57;115) EGP will be determined by steady-state tracer kinetics (57). Arterialized blood will be obtained every 10 min during the last 40 min of the baseline and of each insulin dose for FFA and [6,6-^{2}H]-glucose levels and every 15 min throughout the study for insulin, GH, C-peptide, and glucagon levels.

g. Graded Glucose Infusion. Graded glucose infusion will be performed as previously reported (16;141). Arterialized blood will be obtained every 10 min for 40 min for basal glucose, insulin and C-peptide levels. Glucose will then be infused at 4, 8 and 16 mg/kg/min for 40 min at each level of glucose. Samples will be collected every 10 min during the infusion for glucose, insulin and C-peptide.

h. Glucose-Dependent Arginine Stimulation Test (130). Baseline blood samples will be taken at –5 and –2 min, 10% arginine hydrochloride 5 g (R-Gene 10, Pharmacia, Peapack, NJ) will be infused over 45 s, and samples will be taken at +2, 3, 4 and 5 min. A variable rate infusion of 20% dextrose will be given to increase and maintain blood glucose at 13-15 mmol/L in 20-25 min as in the euglycemic clamp. Baseline sampling, arginine injection 5 g and post-arginine sampling will be repeated. There will be a 2.5 h rest period. Baseline sampling will then be repeated and a high speed infusion (~900 mL/h) of 20% dextrose will be given for 20-25 min to raise the glucose >25 mmol/L as determined by bedside glucose monitoring. Baseline sampling will be repeated, arginine 5 g injected, and post-injection sampling repeated. Samples will be assayed for insulin, C-peptide, proinsulin and glucagon.

i. Frequently Sampled Intravenous Glucose Tolerance Test (121). An FSIGT to determine insulin sensitivity will be performed as reported. In brief, glucose (300 mg/kg) will be injected intravenously at time 0 and tolbutamide, 500 mg iv, will be given at 20 min. Blood samples will be obtained over the next three hours for glucose and insulin levels.

E. HUMAN SUBJECTS

1. **General.** We plan to study ~410 PCOS women, ~170 normal control women, ~80 female offspring of PCOS mothers and ~40 female offspring of control mothers. We anticipate screen failures and subject dropout. Subjects will be between the ages of 18-40 years and in excellent health, with no history of cardiorespiratory, hepatic, or renal dysfunction, and able to give complete informed consent. We will study children between the ages 18-21 years, fetuses, newborns and pregnant women. We will not study any prisoners or institutionalized individuals. Subjects will be disqualified from the study if they develop any serious medical or psychiatric illness.

2. **Sources of Research Material.** Blood will be obtained by venipuncture. Amniotic fluid will be obtained at the time of indicated amniocentesis. DNA will be isolated from blood, fetal cells and placenta (for Project 2). All of these procedures will be performed solely for research purposes.

3. **Recruitment and Consent Procedures.** Subjects will be recruited from the community by advertisement in local newspapers. PCOS subjects will also be referred by their physicians. We have studied more than 100 PCOS women in the last year, and we anticipate no problem in recruiting adequate numbers of PCOS women because of the high prevalence of the condition. Dr. Ehrmann (Consultant) will refer PCOS women from his study population. All subjects will be examined by the PI or a Co-Investigator to determine their eligibility for the studies. Drs. Barnes (Co-I) and Legro (Consultant) will assist in the recruitment of pregnant PCOS women.

Written informed consent will be obtained from each subject before the initiation of any study. There will be no waiver of informed consent. The subjects will be informed of the purpose, duration, specific procedures, risks, and benefits of the study. They will also be informed of their right to withdraw from the study at any time without prejudicing their future care. They will be informed that their anonymity will be maintained and that they will have the right to ask questions of the Investigators or of a patient care representative. They will also be informed of their right to have a copy of their results as soon as the results have become available. Copies of the written informed consent will be given to the participants.

4. Potential Risks

a. Blood withdrawal. The risks associated with blood withdrawal are bruising, bleeding, and phlebitis at intravenous catheter sites. However, these problems are uncommon.

b. Oral Glucose Tolerance Test. The risks associated with this test are the risk of blood withdrawal. Some individuals may experience slight nausea when drinking the glucose solution. There is a slight risk of reactive hypoglycemia with this test.

c. Meal Tolerance Test. The risks associated with this test are the risks of blood withdrawal. Some individuals may experience slight nausea when consuming the meal.

d. ACTH Stimulation Test. The risks of this test include those of blood withdrawal and a possible allergic response to the ACTH (corsyntropin). An allergic reaction is extremely rare.

e. Glucose Clamp and FSIGT Studies. There is a small risk of hypoglycemia during these studies, and testing will be terminated immediately by administration of intravenous 50% dextrose if any signs or symptoms of severe hypoglycemia develop. However, we have performed approximately 200 FSIGTs and 150 euglycemic clamp studies over the past several years and have never had to terminate a study because of hypoglycemia.

f. Arginine Infusion. The risks of an arginine infusion include a possible allergic reaction. An allergic reaction is extremely rare.

g. Amniocentesis. The amniocentesis is clinically indicated, and there is no additional risk from drawing 5 cc of extra fluid.

h. Cord Blood Sampling. This is performed after the delivery of the baby and does not pose an additional risk to the child or to the mother.

i. Antropometric Measurements. There is no risk to the measurements that will be performed.

j. Loss of Privacy. There is potential for loss of privacy.

5. Protection from Potential Risks

i. Disqualification Criteria to Minimize Risk. Subjects will be disqualified from the studies if they have or develop any of the following: weight <50 kg, any general medical illness, hypertension or psychiatric illness.

ii. Blood withdrawal. All participants in studies 1 and 2 will have hemoglobin levels > 12 g/dL. During any 8-week time interval, no subject will have more than ~480 cc of blood withdrawn, the amount which is given at a routine blood donation.

iii. Preparation of Infusates. All infusates will be prepared under sterile conditions by the Research Pharmacist at Northwestern Memorial Hospital. Solutions will be passed through a 0.22 μM filter (Millipore, Bedford, MA) before use. We have performed 200-300 such studies without problems, and no subject has had a febrile reaction to an infusate.

iv. Confidentiality. Coding of data will ensure confidentiality. The subject's name or initials will never be used in any subsequent reporting of the data. Subjects are given three options relating to the use of their DNA: 1) DNA sample may be used for any scientific purpose or project; 2) DNA sample may be used for this project only and other projects with permission; or 3) DNA sample may be used for this project only. Additionally, subjects are given the opportunity to list specific restrictions (if any) regarding the use of their DNA samples.

6. Benefits

Benefits to the Individual Subject. The proposed studies are pure research and not therapeutic. The PCOS women may have IGT or DM2 diagnosed.

Benefits to the Human Community. The objective of this research is to determine the causes of PCOS. PCOS has many long-term health consequences so these studies have substantial public health importance. The risks of these

studies are modest and the potential benefits substantial so the risk:benefit ratio strongly favors performing this research.

F. VERTEBRATE ANIMALS. None.

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H. CONSORTIUM AND CONTRACTUAL AGREEMENTS. None.

I. CONSULTANTS

1. Dr. David Ehrmann, University of Chicago, will assist in the recruitment of PCOS women from his large population of subjects.
2. Dr. Richard S. Legro, Pennsylvania State University, will recruit and study pregnant PCOS women at Hershey Medical Center on a fee-for-service basis for Study 3.

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Thirty-seven candidate genes for polycystic ovary syndrome: Strongest evidence for linkage is with follistatin

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ABSTRACT **Polycystic ovary syndrome (PCOS) is a common endocrine disorder of women, characterized by hyperandrogenism and chronic anovulation. It is a leading cause of female infertility and is associated with polycystic ovaries, hirsutism, obesity, and insulin resistance. We tested a carefully chosen collection of 37 candidate genes for linkage and association with PCOS or hyperandrogenemia in data from 150 families. The strongest evidence for linkage was with the follistatin gene, for which affected sisters showed increased identity by descent (72%; $\chi^2 = 12.97$; nominal $P = 3.2 \times 10^{-4}$). After correction for multiple testing (33 tests), the follistatin findings were still highly significant ($P_c = 0.01$). Although the linkage results for *CYP11A* were also nominally significant ($P = 0.02$), they were no longer significant after correction. In 11 candidate gene regions, at least one allele showed nominally significant evidence for population association with PCOS in the transmission/disequilibrium test ($\chi^2 \geq 3.84$; nominal $P < 0.05$). The strongest effect in the transmission/disequilibrium test was observed in the *INSR* region (*D19S884*; allele 5; $\chi^2 = 8.53$) but was not significant after correction. Our study shows how a systematic screen of candidate genes can provide strong evidence for genetic linkage in complex diseases and can identify those genes that should have high (or low) priority for further study.**

Polycystic ovary syndrome (PCOS) is a common endocrine disorder that is found in ≈4% of women of reproductive age (1) and results in reduced fertility and a 7-fold increased risk for type 2 diabetes mellitus (2). The syndrome is characterized by hyperandrogenism and chronic anovulation. It is also associated with polycystic ovaries, hirsutism, obesity, and insulin resistance. The observation of familial aggregation of PCOS (3–5) is consistent with a genetic basis for this disorder. However, the mode of inheritance of PCOS has not been firmly established. Although some studies support a single dominant gene with high penetrance (6–8), others do not (9).

Several pathways have been implicated in the etiology of PCOS. These include the metabolic or regulatory pathways of steroid hormone synthesis (10, 11), regulatory pathways of gonadotropin action (12), the insulin-signaling pathway (13–15), and pathways regulating body weight (16). Several genes from these pathways have been tested as candidate genes for PCOS (10, 11, 17–23). In particular, in the insulin receptor gene (*INSR*), mutations have been identified in several rare syndromes that, like PCOS, are characterized by hyperandrogenism and insulin-resistant diabetes mellitus. These syndromes include leprechaunism, Rabson–Mendenhall syndrome, and type A syndrome (20–23). Although mutation analysis, linkage studies, and case-control association studies have been carried out with these candidate genes, evidence that any of them play a role in PCOS has not been replicated widely and is still inconclusive. These uncertainties are common in "complex" genetic diseases, where identifying the contributing genes is made difficult by likely genetic heterogeneity, environmental contributions, and multiple etiologies.

As an initial step in the identification of genes playing a role in the etiology of PCOS, we carried out a genetic analysis of 37 candidate genes for PCOS. We chose to analyze candidate genes for PCOS, in part because several well characterized metabolic pathways and candidate genes had been implicated in the etiology of PCOS, but also because we have not yet assembled enough families to carry out a complete genome scan. We tested for linkage with the candidate genes by the affected sib-pair (ASP) test (24), and we tested for association between alleles of the candidate gene markers by the transmission/disequilibrium test (TDT; ref. 25). These methods require no assumption about mode of inheritance, and the TDT, unlike case-control studies, is not influenced by population structure or heterogeneity (26).

MATERIALS AND METHODS

Family Ascertainment and Phenotypes. We studied 150 nuclear families with at least one affected index case. Among the families, 148 were of European origin and 2 were of Caribbean origin. Criteria for diagnosis are described by Legro *et al.* (6). Briefly, an index case was considered affected if she met the following criteria: chronic menstrual irregularity (amenorrhea or ≤ six menses per year; ref. 27) and hyperandrogenemia (HA), i.e., elevated levels of total testosterone or testosterone not bound to sex hormone-binding globulin. Hormone levels were considered elevated if they were more than two standard deviations above the control mean; in our assay these thresholds were 58 ng/dl and 15 ng/dl for total testosterone and testosterone not bound to sex hormone-binding globulin, respectively. Nonclassical 21-hydroxylase deficiency, hyperprolactinemia, and androgen-secreting tumors were excluded (28). HA is a salient and unambiguous biochemical feature of PCOS and is found in a significant proportion of sisters of patients with PCOS, even in the absence of oligomenorrhea (6, 8, 29). Our previous studies have suggested that HA is the major reproductive endocrine phenotype in our families with PCOS (6). For genetic analysis, therefore, female relatives of index cases were considered

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: PCOS, polycystic ovary syndrome; ASP, affected sib pair; HA, hyperandrogenemia; RH, radiation hybrid; STRP, short tandem repeat polymorphisms; cM, centimorgan; TDT, transmission/disequilibrium test; SHGC, Stanford Human Genome Center; IBD, identity by descent.

A Commentary on this article begins on page 8315.

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cted if they had elevated androgen levels, whether or not they had oligomenorrhea (6), and we used the designation "PCOS/HA" to describe this combined category. Female relatives were not screened for nonovarian causes of HA. Women were considered unaffected if they had normal circulating androgen levels, were not taking any confounding medications (e.g., oral contraceptives or insulin-sensitizing agents), and had regular menstrual cycles (menses every 27–35 days; ref. 6). Women not of reproductive age and those not fulfilling the criteria for affected or unaffected phenotypes were assigned the phenotype "unknown" (6). Because the male phenotype corresponding to PCOS is unclear, all men in the study also were assigned the phenotype "unknown."

There were 134 sisters of index cases; 39 sisters were affected (PCOS/HA); 46 sisters were unaffected; and for 49 sisters, the phenotype was unknown. Of the 39 affected sisters, 14 had HA but not oligomenorrhea. Among the 28 multiplex families, the number of sibships with two, three, four, or five affected offspring were 21, 4, 2, and 1, respectively. Maximum sample size for TDT was 163 trios (affected daughter and both parents).

Candidate Genes. We chose 37 candidate genes from four metabolic pathways that have been implicated in the etiology of PCOS (Table 1). These 37 genes map to 33 distinct chromosomal locations. Where possible, we typed polymorphic sites within each candidate gene. For candidate genes without polymorphisms, we chose closely linked short tandem repeat polymorphisms (STRPs). For 28 of the 37 candidate genes, there is at least one polymorphic marker within 1 cM of the candidate gene. For the remaining nine candidate genes, polymorphic markers are 1–4 cM from the candidate gene.

Radiation Hybrid (RH) Mapping. Candidate genes for which accurate mapping information was not available were mapped physically by using the Stanford Human Genome Center (SHGC) medium resolution G3 RH mapping panel (Research Genetics, Huntsville, AL). DNA (40 ng) from each somatic hybrid clone was amplified in a total volume of 8 μl in the presence of 200 μM dNTPs (Amersham Pharmacia), 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.0–2.0 mM $MgCl_2$, 0.36 units *AmpliTaq* polymerase (Roche Molecular Systems, Branchburg, NJ), and 0.5 μM of each primer. The forward primer was labeled with [γ-^{33}P]ATP and samples were electrophoresed on 6% acrylamide, 5 M urea gels at 70 W. Care was taken to choose primers that showed low levels of cross-species homology and, when relevant, low levels of homology to closely related human genes. Genotypes for the RH panel were submitted to a web server (shgc-www.stanford.edu) managed by the SHGC for chromosomal localization. STRP markers were chosen to map as closely as possible to the location determined by RH mapping (see *Results*).

Genotyping. Genotypes were determined at 45 polymorphic loci linked to the 37 candidate genes (Table 1), and the 44 STRPs were assayed by denaturing PAGE. Radioactively labeled primers were used to label 2 of the STRPs (at *AR* and *D11S911*), whereas the remaining 42 STRPs were visualized by using fluorescently labeled primers and the ABI Sequencing system (PE Applied Biosystems). The *HphI* site at the insulin gene *VNTR* is a single nucleotide polymorphism, used as a surrogate for the *VNTR* itself (30), and was assayed by single-strand conformational polymorphism analysis.

For each fluorescently labeled STRP, 45 ng of genomic DNA was amplified as described for the RH mapping, except that the forward primer was fluorescently labeled. For some markers, it was necessary to add 9% (vol/vol) DMSO to obtain suitable PCR product. The STRPs were grouped in five "panels" of eight or nine markers each. The PCR products of any one panel were pooled to give approximately equal signal intensities. Pooled PCR products were electrophoresed in the presence of an internal size standard (Genescan 500) on 4% acrylamide, 5 M urea denaturing gels by using a 377 DNA sequencer (PE Applied Biosystems, Foster City, CA). Genotypes were determined by using the GENESCAN ANALYSIS and GENOTYPER programs (PE Applied Biosystems, Foster City, CA).

The radioactive PCRs were carried out as described for the RH mapping. The PCRs for the *HphI* polymorphism were carried out as described for the fluorescently labeled primers, in the presence of 1.5 mM $MgCl_2$ and [α-^{33}P]dCTP. Samples were electrophoresed overnight at room temperature at 9 W on an MDE gel (FMC).

Statistical Analysis. The extent of identity by descent (IBD) in ASPs was used to test for linkage between the candidate gene and PCOS/HA (24). To incorporate sibships with more than two affected sisters, IBD was calculated by using the weighting scheme described by Suarez and Hodge (31). This method takes into account the fact that the sib pairs in larger sibships are not all independent and sometimes results in fractional numbers of transmitted alleles. The conventional χ^2 statistic calculated with these data is "conservative"; the true significance levels would be more extreme than those quoted. In the present study of 33 independent regions, the apparent significance of any single test will be exaggerated as a result of the multiple tests. The P value for each single test was, therefore, multiplied by 33, and where appropriate, we also report the resulting corrected value P_c. Haplotypes used in multilocus IBD analysis were generated by the GENEHUNTER program (32) when both parents were available. Otherwise haplotypes were reconstructed manually (see below). We tested for association between specific alleles at the candidate gene markers and PCOS/HA by using the TDT (25).

Missing Parental Genotypes. DNA samples could not be obtained from 20 parents. The analysis of sharing in families with one or two missing parental genotypes was done only if the transmissions to the affected could be determined unambiguously and without bias. Genotypes for missing parents were reconstructed by using genotypes of unaffected siblings or those with unknown phenotype. None of these siblings were included in the statistical analysis. Among the 28 multiplex families, there were 4 with one parent missing and 2 with both missing. For the TDT, when one parent was missing, the available parent's genotype was used only if the inheritance could be determined unambiguously and without bias in affected individuals (33, 34).

RESULTS

RH Mapping. RH mapping localized eight candidate genes whose detailed map positions were previously unknown. The results of chromosomal localization, as determined with the SHGC web server, are shown in Table 2. Two-point logarithm of odds scores between the candidate gene and the most closely linked marker ranged from 8.5 (*SHBG*) to 1,000 (*INHA* and *MADH4*), indicating high confidence in the localizations. The markers used for RH mapping were nonpolymorphic expressed sequence tags; using the RH localization, we chose a closely linked highly polymorphic STRP for genotyping. The polymorphic markers used for the genetic analysis are indicated with the approximate map distance between the marker and candidate gene in centimorgans (Table 2).

ASP Analysis. The results of the ASP analysis for all 33 regions are shown in Fig. 1. By far the strongest evidence for linkage was observed for follistatin. The IBD for *D5S623*, the marker mapping closest to follistatin, was 72% (33.8 of 47 transmissions; $\chi^2 = 8.97$; $P = 2.7 \times 10^{-3}$). Haplotypes generated from *D5S623* and two flanking STRPs also showed 72% IBD (47.9 of 66.5 transmissions), but the increase in the number of informative transmissions (from 47 to 66.5) resulted in $\chi^2 = 12.91$ ($P = 3.27 \times 10^{-4}$). Even after correction for multiple testing, this finding remains statistically significant ($P_c = 0.01$). The IBD for the 25 ASPs with PCOS (HA and

Table 1. Genotyping panel for 37 PCOS candidate genes

Marker locus	Gene symbol	Candidate gene	Distance, in centimorgans (cM)*	Chromosomal location
Steroid hormone				
AR	*AR*	Androgen receptor	0	Xq11.2
D15S519	*CYP11A*	CYP11A-cytochrome P450 side-chain cleavage enzyme	0	15q23–24
D15S520	*CYP11A*	CYP11A-cytochrome P450 side-chain cleavage enzyme	0	15q23–24
D10S192	*CYP17*	CYP17-cytochrome P450 17α-hydroxylase/17,20-desmolase	<1	10q24.3
CYP19	*CYP19*	CYP19-cytochrome P450 aromatase	0	15q21
D17S934	*HSD17B1*	17 β-hydroxysteroid dehydrogenase, type I	<2	17q11–21
HSD17B2	*HSD17B2*	17 β-hydroxysteroid dehydrogenase, type II	0	16q24.2
D9S1809	*HSD17B3*	17 β-hydroxysteroid dehydrogenase, type III	<1	9q22
D1S514	*HSD3B1+2*	3 β-hydroxysteroid dehydrogenase, type I and II	<1	1p31.1
D8S1821	*STAR*	Steroidogenic acute regulatory protein	<2	8p11.2
Gonadotropin action				
D12S347	*ACTR1*	Activin receptor 1	<1	12q13.12
D2S2335	*ACTR2A*	Activin receptor 2A	<1	2q22.2
D3S1298	*ACTR2B*	Activin receptor 2B	<1	3p22.2
D5S474	*FS*	Follistatin	<2	5p14
D5S623	*FS*	Follistatin	<0.5	5p14
D5S822	*FS*	Follistatin	<1	5p14
D2S163	*INHA*	Inhibin A	<1	2q33.34
INHBA	*INHBA*	Inhibin β-A	0	7p13–15
D2S293	*INHBB*	Inhibin β-B	2	2cen–2q13
D12S1691	*INHC*	Inhibin C	<1	12q13
D17S1353	*SHBG*	Sex hormone binding globulin	<1	17p13.2
D2S1352	*LHCGR*	Luteinizing hormone/choriogonadotropin receptor	<2	2p21
D2S1352	*FSHR*†	Follicle-stimulating hormone receptor	<2	2p21
D18S474	*MADH4*	Mothers against decapentaplegic homolog 4	<1	18q21
Obesity and energy regulation				
D18S64	*MC4R*	Melanocortin 4 receptor	<3	18q21.32
D7S1875	*OB*	Leptin	0.2	7q31.3–32.1
D1S198	*OBR*	Leptin receptor	0.5	1p31
D2S131	*POMC*	Pro-opiomelanocortin	<1	2p23
D11S911	*UCP2+3*	Uncoupling protein 2+3	<4	11q13
Insulin action				
IGF1	*IGF1*	Insulin-like growth factor I	0	12q22–23
IGF1R	*IGF1R*	Insulin-like growth factor I receptor	0	15q25–26
D7S519	*IGFBP1+3*	Insulin-like growth factor binding protein 1 + 3	1	7p13–7p12
*Hph*I site	*INS VNTR*	Insulin gene VNTR	0	11p15.5
INSR	*INSR*	Insulin receptor	0	19p13.3
D19S216	*INSR*	Insulin receptor	4.2	19p13.3
D19S905	*INSR*	Insulin receptor	0	19p13.3
D19S884	*INSR*	Insulin receptor	1.2	19p13.3
D19S922	*INSR*	Insulin receptor	1.2	19p13.3
D19S391	*INSR*	Insulin receptor	3.6	19p13.2
D19S865	*INSR*	Insulin receptor	7.2	19p13.2
D19S906	*INSR*	Insulin receptor	11	19p13.2
D19S840	*INSR*	Insulin receptor	14	19p13.2
D19S212	*INSL3*	Leydig insulin-like protein 3	<1	19p13.1
D19S410	*INSL3*	Leydig insulin-like protein 3	<1	19p13.1
IRS1	*IRS1*	Insulin receptor substrate 1	0	2q36–37
D3S1263	*PPARG*	Peroxisome proliferator-activated receptor-gamma	<0.2	3p25–24.2

The list contains 45 polymorphic markers closely linked to 37 PCOS candidate genes.
*Distance between polymorphic marker and candidate gene.
†*D2S1352* was used for the two closely linked genes, *LHCGR* and *FSHR*.

menstrual irregularities) did not differ appreciably from that for the 14 ASPs where the nonindex sister had HA alone (data not shown).

We also found a modest increase in sharing at *CYP11A*. IBD was 62% for each of the two markers tested in this region. Haplotypes generated from these markers elevated the IBD to 67% (χ^2 = 5.34). However, after correction for multiple testing, these results were not statistically significant at the P = 0.05 level. For several other markers (*ACTR2A*, *AR*, *INSR*, and *IRS1*), IBD was ≈60%, but in each case, small sample size (≤36 transmissions) led to nonsignificant results.

TDT. The results of the TDT are shown in Fig. 2. Only alleles with at least 10 transmissions from a heterozygous parent to an affected daughter were included in the analysis. There were 349 such alleles. There was evidence for association ($\chi^2 > 3.84$; nominal $P < 0.05$) between at least one allele and PCOS/HA for 14 markers, mapping to 11 candidate genes (*CYP17*, *CYP19*, *HSD17B2*, *IGFBP1+3*, *INHBB*, *INHC*, *INSL3*, *INSR*, *MADH4*, *OB*, and *POMC*). The largest TDT was observed in the *INSR* region with allele 5 of *D19S884* (χ^2 = 8.53; P = 0.004; see Table 3). After correction for 349 tests, however, no alleles had a significantly elevated TDT.

Table 2. RH mapping of candidates genes for PCOS

Gene*	Chromosomal location	Linked marker	Logarithm of odds†	STRP marker for linkage analysis	Distance between STRP and candidate gene, cM
FS	5p14	SHGC-36388	13.8	*D5S623*	<0.5
SHBG	17p13.2	SHGC-35513	8.5	*D17S1353*	<1.0
INHA	2q36.1	SHGC-11864	1,000	*D2S163*	<1.0
INHC	12q13	AFM312XF5	11.5	*D12S1691*	<1.0
ACTR1	12q13.12	AFM298ZB1	9.4	*D12S347*	<0.8
ACTR2A	2q22.2	SHGC-9391	10.3	*D2S2335*	<1.0
ACTR2B	3p22.2	SHGC-115353	9.7	*D3S1298*	<1.0
MADH4	18q21	SHGC-33967	1,000	*D18S474*	<1.0

*Abbreviations are defined in Table 1.
†Two-point maximum logarithm of odds score between candidate gene and most closely linked marker.

Previously Tested Candidate Genes. We tested five gene regions (*INS VNTR*, *CYP11A*, *CYP19*, *CYP17*, and *INSR*) that have been previously tested by others for association or linkage to PCOS. In those studies (10, 11, 35, 36), PCOS was defined by polycystic ovaries (and various associated findings) and premature male pattern baldness (proposed as the male phenotype corresponding to PCOS). Waterworth *et al.* (36) found evidence for linkage with the insulin gene *VNTR* polymorphism (nonparametric linkage score = 3.25; $P = 0.002$). We did not see any significant excess IBD (IBD = 51%) in this region. Our results for this gene and other previously tested genes are shown in Table 3. Waterworth and colleagues (35, 36) also found evidence for association between the insulin *VNTR* and PCOS but only in the form of preferential transmission of the class III allele of the insulin *VNTR* from heterozygous fathers ($\chi^2 = 7.54$; $P = 0.006$), but not from mothers, to daughters with PCOS. In contrast, we saw no evidence for association between the class III alleles of the insulin *VNTR* and PCOS/HA. This finding held for transmissions from both parents to daughters with PCOS/HA or specifically from either fathers or mothers to affected daughters. In fact, there is a nonsignificant excess in the direction opposite to that observed by Waterworth *et al* (36).

Gharani *et al.* (10) found evidence for linkage with the cholesterol side-chain cleavage enzyme, *CYP11A*, (nonparametric linkage score = 3.03; $P = 0.003$). They allowed for genetic heterogeneity and estimated that ≈60% of their 20 families had the linked form. We analyzed two of the STRPs tested by Gharani *et al.* (ref. 10; *D15S519* and *D15S520*) and found modest evidence for linkage (see above).

Gharani *et al.* (10) also found an association with *D15S520*, which is located in the promoter region of *CYP11A*. They found that, compared with controls, allele 5 of *D15S520* was seen significantly less often in affected women ($P = 0.03$) and in women with elevated androgen levels alone ($P = 0.002$). In our families, there was no significant association between PCOS/HA and any alleles at this marker or the closely linked *D15S519*; allele 5 of *D15S520* was transmitted at a slightly reduced frequency (72:82), but the difference was not statistically significant.

Gharani *et al.* (10) were able to exclude linkage with *CYP19*. We also found no significant evidence for linkage in this region (IBD = 51%). There were two alleles with elevated TDT (allele 6, $\chi^2 = 4.35$; allele 7, $\chi^2 = 6.67$), but after correction for multiple testing, these findings were no longer statistically significant.

Like Carey *et al.* (11), we found no evidence for linkage between *CYP17* and PCOS/HA (IBD = 49%). Carey *et al.* (11) did find evidence for association with a variant nucleotide in the *CYP17* promoter region, although these findings did not remain significant when more patients were added to the analysis (37). We found that one allele (allele 10 of *D10S192*) in the *CYP17* region does have a somewhat elevated TDT ($\chi^2 = 6.87$), but after correcting for multiple testing, this finding was not statistically significant.

Several studies have sought, but failed to find, mutations in the *INSR* coding region of patients with PCOS (14, 17, 19, 21–23). Our findings in this region are consistent with previous

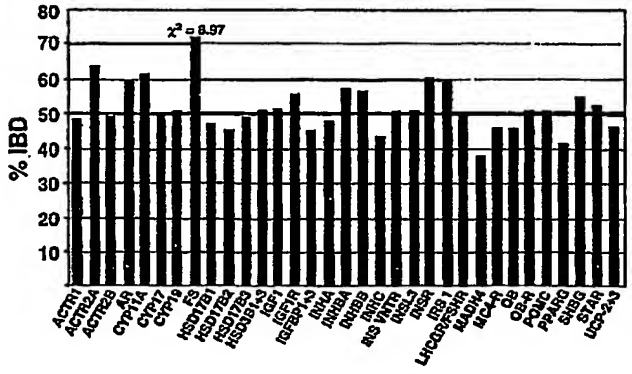

FIG. 1. Summary of ASP analysis. IBD results for the marker with the highest IBD in each candidate gene region are shown. IBD was calculated for 39 ASPs in 28 families at each of 33 candidate gene regions (*x* axis). The IBD expected under the null hypothesis of no linkage is 50%. The only χ^2 value > 3.84 (critical value for $P < 0.05$) is for follistatin.

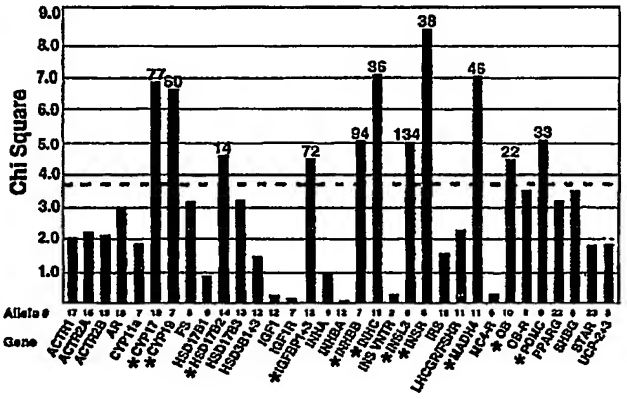

FIG. 2. Summary of TDT analysis. The dashed line indicates a χ^2 value of 3.84 ($P = 0.05$). The candidate gene regions and the allele with the highest χ^2 value for each region are listed on the *x* axis. Each allele with a nominally elevated χ^2 (>3.84) is indicated with an asterisk, and the number of transmissions tested is shown above the bar. A total of 349 alleles at 45 loci were tested.

Table 3. Results from the present study for linkage and TDT analysis of genes previously tested in other studies

Gene (ref.)	ASP Analysis: IBD	Not IBD	Total, *n*	IBD, %	χ^2	*P*	TDT: Allele	Transmitted	Not transmitted	χ^2	*P*
INS VNTR (35,36)							Class III				
	15.2	14.8	30	51	0.00	>0.5	Total	50	54	0.15	>0.5
							Paternal	23	24	0.02	>0.5
							Maternal	27	30	0.16	>0.5
CYP11A (10)											
D15S519	22.8	14.2	37	62	2.03	0.15	7	73	90	1.77	0.18
D15S520	21	12.7	33.7	62	2.06	0.15	5	72	82	0.65	0.42
Haplotype*	30.8	15.2	46	67	5.34	0.02	—	—	—	—	—
CYP19 (10)											
	25.9	24.7	50.7	52	0.03	<0.5	6	56	36	4.35	0.04
							7	20	40	6.67	0.01
CYP17 (11,37)											
D10S192	28.5	29.5	58	49	0.02	>0.5	10	27	50	6.87	0.01
INSR (18–20)											
INSR	17.5	12.5	30	58	0.83	0.36	13	39	21	5.40	0.02
D19S884	27.3	24.7	52	53	0.14	>0.5	5	10	28	8.53	0 004
Haplotype†	34.7	29.9	64.7	54	0.36	>0.5	—	—	—	—	—

**D15S519–D15S520.*
†pter*D19S905–INSR–D19S884–D19S922*cen.

studies in that we also do not find evidence for linkage between PCOS and the *INSR*. IBD for the *INSR* region ranges from 53% at *D19S884* to 61% at *D19S922*; neither is statistically significant, and IBD for the much more informative 1.2-cM haplotype for this region (65 transmissions) is only 54% (χ^2 = 0.36). We did, however, find evidence for association (elevated TDT) in the *INSR* region. The strongest evidence for association is with allele 5 of *D19S884*; however, this finding is not statistically significant after correction.

DISCUSSION

We tested for linkage and association between 37 candidate genes and PCOS/HA in data from 150 families, including 39 affected sister pairs. The phenotype PCOS/HA was defined by HA and oligomenorrhea in index cases and HA with or without oligomenorrhea in affected sisters. We found evidence for linkage with two genes: follistatin and *CYP11A*. Only the linkage with follistatin remains significant after correction for multiple testing.

Both of these regions are worthy of follow up studies. The cholesterol side-chain cleavage enzyme *CYP11A* converts cholesterol to pregnenolone, a rate-limiting step of steroidogenesis. A mutation that causes up-regulation of *CYP11A* activity could therefore result in an increase in androgen levels, one of the criteria used to define affected status in this study (6). The evidence for linkage with *CYP11A* (Table 3) was not very strong when each marker was considered separately, but when we assessed IBD by considering sharing of the haplotype defined by *D15S519–D15S520* (a span of <1 kb), the IBD was 67% of 46 transmissions, and the corresponding χ^2 was 5.34 (nominal P = 0.02). However, these results are no longer significant after correction for multiple testing (multiplying the P value by 33, the number of regions tested). Because Gharani *et al.* (10) also found evidence for linkage with *CYP11A*, our findings are, to some extent, a confirmation. In this situation, multiplying by the full 33 tests probably provides a correction that is too stringent, but it is not known what correction should be used instead.

By far the most convincing evidence for linkage was found with follistatin. Follistatin, an activin-binding protein, neutralizes the biological activity of activin *in vitro* and *in vivo* (38, 39). Activin, a member of the transforming growth factor-β superfamily, and follistatin are expressed in numerous tissues, including the ovary, pituitary, adrenal cortex, and pancreas. Activin promotes ovarian follicular development, inhibits thecal-cell androgen production, and increases pituitary follicle-stimulating hormone secretion and insulin secretion by pancreatic β-cells (39, 40). An increase in level or in functional activity of follistatin might, therefore, be expected to arrest follicular development, increase ovarian androgen production, reduce levels of circulating follicle-stimulating hormone, and impair insulin release. These changes are all characteristic features of PCOS (3, 41). Indeed, overexpression of follistatin in transgenic mice results in suppression of serum levels of follicle-stimulating hormone and arrested ovarian folliculogenesis (38).

With 66 transmissions of informative haplotypes at the follistatin locus, the finding of 72% IBD is highly significant, even after correction for 33 tests (P_c = 0.01). Although, in principle, some gene other than follistatin could give rise to the evidence for linkage of PCOS/HA with this region, we have focused on follistatin, because it is the candidate gene that led us to study this region.

We also tested for association in the follistatin and *CYP11A* regions. No allele of any marker in these regions showed significant evidence for allelic association. Although an allelic association detected by the TDT would have provided support for linkage, the absence of association is not inconsistent with linkage, because the effect detected by the TDT requires linkage disequilibrium in addition to linkage. It follows that genetic markers may reveal linkage without showing allelic association with the disease, especially if, as in the case of follistatin, the marker is not extremely tightly linked (Table 1).

We carried out a very complete analysis of association by the TDT for all markers, because this type of analysis is a particularly appropriate test for a possible role of a candidate gene. However, the very large number of alleles tested (349) makes it difficult to interpret nominally significant results. Furthermore, 6 of the 11 nominally significant tests shown in Fig. 2 are based on a relatively small number of transmissions (<50). Even for the larger samples with χ^2 values close to 7

CYP17 and *CYP19*), we are uncertain about the ultimate significance of the associations we observed. The strongest evidence for association was seen with allele 5 of *D19S884* ($\chi^2 = 8.53$; $P = 0.004$; not significant after correction). *D19S884* was chosen as a marker for the insulin receptor, considered a candidate gene on the basis of several previous studies (13, 21, 41). Nevertheless, the results are not conclusive, in part because of the modest sample size, and larger independent samples will be needed for a convincing replication of these findings.

In the present study, we have carried out analyses of genetic linkage and population association for a set of candidate genes for PCOS. We show how these genetic analyses can be used to screen a large number of candidate genes, without testing each gene for mutation(s). These approaches identify the candidate genes with the strongest evidence for genetic linkage and suggest which genes make minimal contributions to the etiology of the disease. The alternative procedure of screening one candidate gene at a time for mutations contributing to such diseases would be very inefficient, because variants that predispose to disease are heterogeneous and common in complex diseases such as PCOS. On the other hand, combined analysis of linkage and association can provide evidence that one (or several) candidate genes contribute to susceptibility, even though the precise genetic variant is not known. Such genetic evidence can then be used to guide further studies of those candidate genes. Our results suggest that variation at or near the follistatin gene contributes to the HA of PCOS.

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